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A NOVEL METHOD FOR THE DECREASE OF PHENOLIC CONTENT IN COMMERCIAL CANOLA MEAL USING AN ENZYME PREPARATION SECRETED BY THE WHITE-ROT FUNGUS *TRAMETES VERSICOLOR*

by

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Abstract

Canola meal (CM) is a by-product from the deoiling process of canola seeds. Due to its favourable nutritional composition, it is used as a supplementary source of nutrients in live-stock diets. Furthermore, its amino acid composition can prove useful in the preparation of high quality protein isolates for human consumption, if some concerns related to the presence of anti-nutritional components such as glucosinolates, phytate and phenolics are solved.

This research project was focused on the development of a novel enzymatic method for upgrading commercial canola meal by decreasing its phenolic content. The new method is based on the enzymatic transformations of sinapic acid esters (SAE) and free sinapic acid (SA), the two main constituents of the phenolic fraction of CM, using an enzyme preparation secreted by the white-rot fungi *Trametes versicolor* ATCC 42530. The study was divided into three parts: (1) enzyme production; (2) characterization of the enzyme produced - a model enzymatic system; (3) enzymatic upgrading of commercial canola meal - a canola meal system.

The enzyme preparation used in this work was produced by a submerged growth process, in batch reactors at ambient temperature using a semi-defined liquid medium. The effects of the initial pH of the medium, the initial concentrations of glucose, inoculum, and Vogel's minerals were investigated. It was found that the presence of the Vogel's minerals was essential for enzyme production. The enzyme preparation with the highest activity was obtained using a medium containing 0.5% w/v of the minerals. The enzyme production depended on the initial pH of the medium. The increase in the initial biomass concentration accelerated enzyme production, however, the optimum amount of inoculum should be used to obtain the highest enzyme yield. The production of the enzyme preparation was inhibited by the glucose concentrations higher than 1.1% (w/v). The results obtained in this part of the project led to the formulation of the growth conditions which resulted in shortening the time of the process by 50% compared to the methods reported in the literature.

The enzyme preparation produced was characterized in the model enzymatic system using sinapic acid (SA), sinapine (SIN) and sinapaldehyde (SALD). The results showed that the enzyme is inhibited by high concentrations of SIN and SA. Depending on the substrate, the optima temperatures and pH are in the range of 50°C-60°C and 3.3-4.5, respectively. The enzyme was very thermostable; the maximum thermal stability was noticed at pH values between 4.5 and 6.5. Sodium azide was a strong inhibitor of this enzyme while its activity was not affected by EDTA. Based upon the results obtained, the enzyme was characterized as a polyphenol oxidase.

The transformation of SA was studied in more detail. It was found that this reaction proceeded according to the Theorell-Chance Bi-Bi mechanism, with oxygen being the first substrate. It was also shown that the overall enzymatic conversion of SA took place in two distinct stages, Phase A and Phase

B. In Phase A, the actual transformation of SA occurred with the formation of dehydrodisinapic acid dilactone, DAD. The enzymatic formation of DAD has never been reported. The DAD was identified based on the proton and carbon NMR, mass spectroscopy (MS), and Fourier transformed infrared spectrophotometry (FTIR) data, and its identity was confirmed by X-Ray crystallography. Phase B of the enzymatic transformation of SA, the beginning of which was marked by the complete disappearance of SA, involved the enzymatic conversion of DAD with the formation of several intermediate products, some of which were separated and analyzed using (MS) and (FTIR). The final, organic-solvent extractable/soluble product of the overall transformation of SA was 2,6-dimethoxy-*p*-benzoquinone. After a 96 hour reaction, its yield was 23.2%. This compound was also detected during the enzymatic transformation of SIN, as well as during the treatment of CM.

The overall transformation of SA was described by a mathematical model based on the Theorell-Chance Bi-Bi mechanism with oxygen as the first substrate, and the second substrate being one of the alternate phenolic compounds: SA, DAD and the unknown compound formed *via* the thermolysis of DAD. The enzyme's kinetic parameters in the model were estimated using the data collected in the experimental setup, designed and built in this study, in which the effects of temperature and oxygen concentration on the rate of enzymatic transformations could be independently analyzed. The model simulation results led to the recommendations for the experimental conditions for the examination of the effect of temperature on the enzymatic reaction involving oxidases and oxygenases.

The modeling of sinapine transformation was also considered in this work. A mechanistic model, based on the Theorell-Chance Bi-Bi mechanism, that simultaneously predicted the effects of the concentrations of the enzyme, SIN and oxygen, for any given pH and temperature level, on the rates of the enzymatic reaction was formulated. The model parameters were estimated following the new procedure developed in this work for analyzing data concerning the effects of pH and temperature on enzyme activity. The formula relating the optimum pH of the reaction with the temperature was proposed.

The enzyme preparation produced and subsequently characterized was used in the novel method to decrease the phenolic content in commercial CM, which was based on the addition of the enzyme to the meal-buffer slurry. A 98% decrease in the concentration of SAE, from their initial concentration of 22.2 g/kg to 0.44 g/kg, was observed after 1 hour of the treatment. The content of free sinapic acid dropped below the detection limit, whereas the concentration of the insoluble-bound sinapic acid esters was decreased by 56%. The tannin content in the treated meal was also reduced from the original level by 50%. Several process variables were considered for optimizing the process, namely: pH, temperature, enzyme, meal and oxygen concentrations. It was found that: (1) the natural buffering capacity of CM resulted in the negligible effect of the pH of the buffer, which was used as the continuous phase in the process, on the extent of SAE decrease; (2) the system was saturated with the enzyme when the enzyme concentration was 4 nkat per mL of the continuous phase; (3) the optimum temperature was 50°C. The process could be carried out with the meal concentration as high as 43% w/v, even at temperatures as high

as 100°C, due to the protective action of canola meal particles and canola meal proteins that caused an increase in the thermal stability of the enzyme. The half-life of the enzyme incubated in the presence and absence of CM were, 45 and 11 hours, respectively, at 50°C.

The enzymatic process for upgrading the meal was described by the mathematical model describing the extraction of the SAE from the meal into a solution of finite volume in which the enzymatic reaction took place. The solution to this problem was obtained using *Duhamel's theorem*. It was found that in order to describe the investigated process, CM had to be considered as a mixture of two types of particles, both having the same diameter but different effective diffusivities. This approach allowed for the description of the final stages of the process, which were limited by the extraction of SAE from the meal. The parameters in the model were estimated based on the experimental results regarding the effects of the concentration of the enzyme and oxygen, and the effect of the process temperature. The general set of parameters that can be used to describe the process at any set of desired conditions was given.

A few modifications of the proposed enzymatic method were considered. In one, the meal was treated with the polyphenol oxidase in the presence of the cell-wall material solubilizing enzymes. This treatment resulted in a faster decrease in SAE content due to the increase in the rate of intraparticle diffusion of the SAE. In another modification, only highly concentrated meal-buffer slurries were treated with enzyme in order to minimize the protein loss encountered during the basic treatment. In yet another process variation, the aqueous continuous phase (*i.e.*, buffer) was replaced with hexane to examine the possibility of implementation of the enzymatic step into the deoiling processes of canola seeds. In this case, the enzymatic treatment had also proved effective in reducing the phenolic content in CM.

A direct comparison of the new enzymatic method with other methods reported in literature for the decrease in the phenolic content of rapeseed meals was performed. It was found that among the methods tested, the enzymatic treatment was the most effective, followed by the lime treatment.

Evaluation of the properties of the protein isolates obtained from the enzymatically treated meals showed that the enzymatic process did not reduce the value of the proteins, as compared to the isolate obtained from the untreated meal. In fact, after the addition of a simple acetone-washing step the isolate from the enzymatically treated meal had better properties.

Some recommendations for further research concerning the upgrading of CM by reducing its phenolic content using enzymes of the white-rot fungus *Trametes versicolor* are given.

Résumé

Les tourteaux de canola (CM) sont des sous-produits de procédé de pressage des graines oléagineuses de canola. Due à ses compositions nutritives favorables, ils sont utilisés comme source supplémentaire des nutriments dans l'alimentation animale. De plus, ses compositions en acide aminé peuvent se montrer utile dans la préparation des protéines de haute qualité pour la consommation humaine, à condition que les problèmes reliés à la présence des composés nutritifs, comme les glucosinolates, les phytate et les phénoliques, soient résolus.

Le but de ce projet de recherche était le développement d'une nouvelle méthode enzymatique en vue de l'amélioration de la qualité des tourteaux commerciaux de canola en diminuant ses compositions phénoliques. La nouvelle méthode est basée sur les transformations enzymatiques des deux constituants principaux de la fraction phénolique des tourteaux de canola, notamment les estères de l'acide sinapique (SAE) et l'acide sinapique (SA), en utilisant une préparation d'enzyme sécrété par le white-rot fungus *Trametes versicolor* ATCC 42530. L'étude comprenait trois parties: (1) production d'enzyme; (2) caractérisation de l'enzyme produit - un système enzymatique modèle; (3) amélioration de la qualité des tourteaux commerciaux de canola - un système réel de canola.

La préparation d'enzyme utilisée dans ce travail a été produite par un procédé de croissance submersible, dans des réacteurs semi-contenus à la température ambiante en utilisant un milieu liquide sema-défini. Les effets de la valeur initiale de pH du milieu, les concentrations initiales de glucose, inoculum, et les minéraux de Vogel ont été étudiés. L'étude a montré que la présence des minéraux de Vogel était essentielle pour la production d'enzyme. L'enzyme ayant une activité plus élevé a été obtenue en utilisant un milieu contenant une concentration de 0.5 % m/v des minéraux. La production d'enzyme dépendait de la valeur initiale de pH du milieu. L'augmentation de la concentration initiale de biomasse accélérait la production d'enzyme, cependant, la quantité optimum de l'inoculum doit être utiliser pour obtenir le rendement le plus élevé d'enzyme. Les résultats obtenus dans cette partie du projet ont abouti à la formulation des conditions de croissance qui réduisaient par 50 % le temps du procédé en comparaison avec les méthodes rapportées dans la littérature.

La préparation d'enzyme produite a été caractérisée dans le système enzymatique modèle en utilisant l'acide sinapique (SA), la sinapine (SIN) et l'aldéhyde de l'acide sinapique (SALD). Les résultats ont montré que l'enzyme est inhibité par des hautes concentrations de SIN et SA. Les températures et les valeurs du pH optimales, qui dépendaient de substrat, se trouvaient respectivement dans les intervalles de 50 à 60 C et 3.3 à 4.5. L'enzyme était très thermiquement stable; la stabilité thermique maximale a été obtenue dans un intervalle du pH de 4.5 à 6.5. L'azide de sodium était un inhibiteur très fort de cet

enzyme pendant que son activité n'a pas été affectée par l'EDTA. En se basant sur les résultats obtenus. l'enzyme a été caractérisé comme étant l'oxydase de polyphenole.

Une étude plus approfondie de la transformation de SA a été menée. On a trouvé que cette réaction s'est déroulée selon le Bi-Bi mécanisme de Theorell-Chance ou l'oxygène est le substrat principal. On a trouvé aussi que la conversion enzymatique globale de SA est effectuée en deux étapes distinctes. Phase A et Phase B. Dans la Phase A, la transformation actuelle de SA a eu lieu avec la formation de l'acide dehydrodisinapique dilactone (DAD). La formation enzymatique de DAD n'a été jamais rapportée dans la littérature. La DAD a été identifié par les méthodes de NMR de proton et de carbone, la spectroscopie massique (MS) et la spectrophotométrie infrarouge (FTIR) et son identité a été confirmée par la cristallographie par rayons X. La Phase B de la transformation enzymatique de SA dont dès le début, la transformation est marquée par la disparition complète de SA, impliquait la conversion enzymatique de DAD avec la formation de plusieurs produits intermédiaires, dans les quels quelques produits ont été séparés et analysés avec les méthodes MS et FTIR. Le produit final de la transformation globale de SA selon la méthode de l'extraction au solvant organique est identifié comme le 2,6-dimethoxy-*p*-benzoquinone. Après 96 heures de réaction, son rendement était de 23.2 %. Ce composé a été aussi détecté lors de la transformation enzymatique de SIN et celui de traitement des tourteaux de canola.

La transformation globale de SA a été décrit par un modèle mathématique basé selon le Bi-Bi mécanisme de Theorell-Chance avec l'oxygène comme substrat principal et l'un des composés alternatifs phénoliques: SA, DAD et le composé inconnu formé par thermolyse de DAD, comme substrat secondaire. Les paramètres cinétiques de l'enzyme dans le modèle ont été estimés en utilisant les données expérimentales dans les quelles les effets de la température et de la concentration d'oxygène sur le taux de réaction des transformations enzymatique peuvent être analyse indépendamment. Les résultats de la simulation numérique du modèle ont conduit aux recommandations des conditions expérimentales pour l'examen de l'effet de température sur la réaction enzymatique impliquant les oxydases et les oxygenases.

La modélisation de la transformation de sinapine a été aussi considérée dans cette étude. Un modèle théorique a été développé en se basant sur le Bi-Bi mécanisme de Theorell-Chance et qui prédirait simultanément les effets de la concentration de l'enzyme, SIN et l'oxygène sur les taux de la réaction enzymatique à de niveau de pH et de température donnes. Les paramètres du modèle ont été estimés selon la nouvelle procédure développé au cours de cette étude pour l'analyse des données concernant les effets de pH et de température sur l'activité de l'enzyme. Une formule reliant le pH optimal de la réaction avec la température a été proposée.

La préparation enzymatique ainsi produite et ultérieurement caractérisée a été utilise dans la nouvelle méthode pour réduire le contenu phénolique dans les tourteaux de canola commerciaux. Cette opération a été basée sur l'addition de l'enzyme à la solution tampon des tourteaux. Une réduction de 98% de la concentration de SAE, a partir d'une concentration initiale de 22.2 g/Kg, a été observe après une heure de traitement. Le contenu en acide sinapique libre était au-dessous de la limite de détection, alors

que la concentration de l'ester de l'acide sinapique insoluble a été réduite par 56%. Le contenu des tanins dans les tourteaux traites a été aussi réduit de son niveau initial par 50%. Plusieurs variables du procédé ont été considérés en vue de l'optimisation du procédé, notamment: pH, température, enzyme, tourteaux et concentrations d'oxygène. On a trouve que: (1) la capacité tampon naturelle des tourteaux de canola rendait négligeable l'effet de pH de la solution tampon, utilisée comme phase contenue dans le processus, sur l'étendue de la diminution de SAE; (2) le système a été sature par l'enzyme quand la concentration de celui ci dans la phase contenue a été de 4 nkat par mL; (3) La température optimale a été 50 C. Le procédé peut être mis a exécution en utilisant une concentration des tourteaux aussi élevée que 43% m/v, même à des températures aussi élevée que 100 C, ceci est dû à l'action protectrice des particules des tourteaux de conola et des protéines des tourteaux de canola qui causait l'augmentation de la stabilité thermique de l'enzyme. Le demi-temps de vie de l'enzyme incube en présence et en absence des tourteaux de canola à 50 C étaient de 45 et 11 heurs respectivement.

Le procédé enzymatique pour l'amélioration des tourteaux a été décrit par un modèle mathématique qui décrivait l'extraction de SAE des tourteaux dans une solution de volume fini dans la quelle la réaction enzymatique se déroulait. Le théorème de Duhamel a été utilisé pour trouver la solution pour ce problème. On a trouve que pour décrire le procédé en question, CM devrait être considéré comme un mélange de deux types des particules, les deux ayant le même diamètre mais des diffusivites effectives différentes. Cette approche permettrait la description des stages finaux de procédé qui étaient limités par l'extraction de SAE à partir des tourteaux. Les paramètres du modèle ont été estimés en se basant sur les résultats expérimentaux relativement aux effets de la concentration de l'enzyme et de l'oxygène, et à l'effet de la température du procédé. L'ensemble global des paramètres qui peuvent être utiliser pour décrire le procédé pour n'importe quel ensemble des conditions a été présenté.

Quelques modifications concernant la méthode enzymatique proposée ont été considérées. Dans l'une, les tourteaux ont été traites par l'oxydase de polyphenol en présence de matière solubilisant des enzymes. Ce traitement avait comme résultat une diminution plus rapide de contenue de SAE due à l'augmentation de taux intra-particule de diffusion de SAE. Dans une autre modification, les suspensions tampon des tourteaux à haute concentration ont été traitées par l'enzyme à fin de minimiser la perte des protéines lors du traitement basique. Néanmoins dans une autre variation du procédé, la phase contenue aqueuse (tampon) a été remplacée par l'hexane pour examiner la possibilité d'implémentation de l'étape enzymatique dans le procédé de pressage des graines oléagineuses de canola. Dans ce cas, le traitement enzymatique se montrait efficace dans la réduction des composes phénolique des tourteaux de canola.

Une étude comparative directe de la nouvelle méthode enzymatique avec les méthodes existantes dans la littérature pour la diminution de contenu phénolique a été faite. Parmi ces méthodes, il s'est avéré que la méthode de traitement enzymatique était la plus efficace suivie par le traitement aux chaux.

L'évaluation des propriétés des extraits de protéine obtenus à partir des tourteaux traites par la méthode enzymatique ont montré que le procédé enzymatique n'avait pas d'effet sur la valeur nutritive

des protéines en comparaison avec les tourteaux non traites. En effet, leurs propriétés se sont améliorées par simple lavage à l'acétone.

Quelques recommandations pour une étude de recherche ultérieure concernant l'amélioration de la qualité des tourteaux de canola par les enzymes de White-rot fungus *Trametes versicolor* sont données.

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Nomenclature

ABBREVIATIONS

ATCC	- American Type Culture Collection
a.c.u.	- arbitrary concentration units
BQ	- 2,6-dimethoxy-benzoquinone
ChS	- Chemical Spectrophotometric method
CM	- Canola Meal
CPC	- Canola Meal Protein Concentrate
DAD	- Dehydroxydisinapic Acid Dilactone
DS	- Direct Spectrophotometric method
FA	- Ferulic Acid
FIN	- Iso-Ferulocholine
GLC	- Gas Liquid Chromatography
GRAS	- Generally Recognize As Safe
HPLC	- High Performance Liquid Chromatography
FPU	- Filter Paper Unit
FTIR	- Fourier Transformed Infrared Spectroscopy
IB-SAE	- Insoluble-Bound Sinapic Acid Esters
LC-MS	- Liquid Chromatography-Mass Spectroscopy
MeOH	- Methanol
MeCl	- Methylene Chloride
MS	- Mass Spectroscopy
MSC	- Model Selection Criterion
NMR	- Nuclear Magnetic Resonance spectroscopy
PDA	- Photo Diode Array detector
PPO	- Polyphenol Oxidase
RCM	- Rapeseed/Canola Meal
SA	- Sinapic Acid
SAE	- Sinapic Acid Esters
SALD	- Sinapaldehyde
SALC	- Sinapyl alcohol
SIN	- Sinapine
SSA	- Steady State Approximation

TAN - Tannin

TCA - Trichloroacetic Acid

TLC - Thin Layer Chromatography

TMA - Trimethylamine

UV-VIS - Ultra-Violet Visible

XU - Xylanase Unit

SYMBOLS

A_{ini} - initial absorbance ..... [-]

$A_{initial}$ - initial absorbance for ChS method ..... [-]

A_{final} - final absorbance for ChS method ..... [-]

A_t - final absorbance ..... [-]

ΔA_i - constant specific for i^{th} reaction ..... [-]

ΔB_i - constant specific for i^{th} reaction ..... [K^{-1}]

ΔC_i - constant specific for i^{th} reaction ..... [K^{-2}]

ΔD_i - constant specific for i^{th} reaction ..... [K^{2}]

ΔG_i^0 - standard Gibbs energy of reaction i ..... [J mol^{-1}]

ΔH_O - enthalpy of reaction ..... [kJ mol^{-1}]

ΔS^* - entropy change ..... [J mol^{-1} K^{-1}]

Δt - enzymatic assay time ..... [s]

$[O_2]$ - oxygen concentration ..... [mM]

[SA] - sinapic acid concentration ..... [mM]

[SIN] - concentration of sinapine ..... [mM]

[DAD] - concentration of DAD ..... [mM]

[XV] - concentration of XV ..... [mM]

$B_{iso,i}$ - coefficient dependent on $K_{iso,i}$ ..... [mL_{liquid}/g_{solid}]

$C_{O_2}^l$ - concentration of oxygen in the bulk liquid (canola meal system) ..... [mol mL^{-1}]

$C_{O_2}^g$ - concentration of oxygen in the gas phase (canola meal system) ..... [mol cm^{-3}]

$C_{P_{Cat}}$ - concentration of the products, PCM, in the bulk liquid (canola meal system) ..... [mol mL^{-1}]

$C_{O_2}^*$ - equilibrium concentration of oxygen at the gas-liquid interface ..... [mol mL^{-1}]

C_0^T - initial concentration of the active-native form of the enzyme ..... [a.c.u.]

C_{SAE}^{rec} - recalculated concentration of SAE ..... [g kg^{-1}]

C_{SAE}^{buffer} - concentration of SAE after treatment with the deactivated enzyme at given condition .. [g kg^{-1}]

C_{SAE}^{sl} - sensitivity limit for ChS method [g kg^{-1}]

C_{DS} - concentration of SAE measured using DS method ..... [g kg^{-1}]

C_{ChS} - concentration of SAE measured using ChS method ..... [g kg^{-1}]

C_{HPLC} - concentration of SAE measured using HPLC method ..... [g kg^{-1}]

C_{SEA} - concentration of the extracted SAE in the bulk liquid (canola meal system) ..... [mol mL^{-1}]

$c(t)$ - concentration of the solute in the sphere after time t of extraction ..... [a.c.u.]

$\bar{c}(t)$ - average concentration of the solute in the sphere after time t of extraction ..... [a.c.u.]

$\bar{c}_{\infty}$ - average concentration of the solute in the sphere after infinite time of extraction ..... [a.c.u.]

c_0 - initial concentration of the solute in the solid ..... [a.c.u.]

c_{∞} - concentration of the solute in the solid after infinite time..... [a.c.u.]

$D_{eff,i}$ - Effective intraparticle diffusivity of the solid in the i^{th} fraction ..... [$m^2 s^{-1}$]

Dil - enzyme dilution factor ..... [-]

E^- - initial form of enzyme

E^{2-} - deprotonated form of enzyme

E^-_a - active form of protonated enzyme

E^-_d - thermally deactivated form of enzyme

E^0 - protonated form of enzyme

E - active form of the enzyme ..... [a.c.u.]

E_0 - initial concentration of the active enzyme in the canola meal system ..... [mM]

E_{CM} - concentration of active enzyme in the canola meal system ..... [M]

E_d - energy of enzyme deactivation ..... [kJ mol^{-1}]

$E_{i,a}$ - activation energy for reaction i ..... [kJ mol^{-1}]

E^p - active protected form of the enzyme ..... [a.c.u.]

E_p - active protected form of the enzyme (canola meal system) ..... [M]

E_t - total enzyme concentration (canola meal system) ..... [mM]

e_0 - total initial concentration of the enzyme ..... [mM]

e_0 - enzyme concentration of the stock solution ..... [%]

e_0^- - initial concentration of the active protonated form of the enzyme ..... [mM]

$e(t)$ - enzyme concentration after time t of incubation ..... [a.c.u.]

$e_a^-(t)$ - concentration of active form of the protonated enzyme after time t of incubation ..... [mM]

$\langle e_a^- \rangle$ - average concentration of the active form of enzyme during the reaction ..... [mM]

$f(w_i^*)$ - function representing sorption isotherm ..... [$g_{solute}\, g_{solvent}^{-1}$]

h - Planck constant ..... [J s]

h^+ - concentration of hydrogen ions ..... [M]

h_{opt}^{+} - optimum concentration of hydrogen ions ..... [M]

I - inactive form of the enzyme

I_i - constant specific for i^{th} reaction ..... [-]

j - identifier for CM fraction of a given size ..... [-]

J_i - constant specific for i^{th} reaction ..... [J mol^{-1}]

K_1 - deprotonation dissociation constant ..... [M]

K_2 - protonation dissociation constant ..... [M]

K_i - inhibition constant (enzyme production) ..... [mL mg^{-1}]

$K_{i,A}$ - inhibition constant for compound A ..... [mM]

$K_{i,O2}$ - inhibition constant for oxygen ..... [mM]

$K_{i,O2,CM}$ - inhibition constant for oxygen (canola meal system) ..... [mol mL^{-1}]

$K_{uo,i}$ - partition coefficient defined as $K_{uo} = \frac{w_i}{w_i^e}$ ..... [$mL_{liquid}\ g_{solid}^{-1}$]

$K_{m,j}$ - Michaelis-Menten constant for compound j = A; B; C; D ..... [mM]

$K_{m,j}^{app}$ - apparent Michaelis-Menten constant for compound j ..... [mM]

$K_{m,O2}$ - Michaelis-Menten constant for O_2 ..... [mM]

$K_{m,SA}$ - Michaelis-Menten constant for SA ..... [mM]

$K_{m,SIN}$ - Michaelis-Menten constant for SIN ..... [mM]

$K_{m,DAD}$ - Michaelis-Menten constant for DAD ..... [mM]

$K_{m,XV}$ - Michaelis-Menten constant for XV ..... [mM]

K_{L_x} - Langmuir constant ..... [g mL^{-1}]

K_S - substrate concentration at which productivity reaches half its maximum ..... [mg mL^{-1}]

$K_{O2,CM}$ - Michaelis-Menten constant for O_2 (canola meal system) ..... [mol mL^{-1}]

K_{SAE} - Michaelis-Menten constant for SAE (canola meal system) ..... [mol mL^{-1}]

K_{PCM} - Michaelis-Menten constant for P_{CM} (canola meal system) ..... [mol mL^{-1}]

$k_{0,i}$ - frequency factor for i^{th} rate constant ..... [$mM^{1-no}\ s^{-1}$]

k_{-1} - first order rate constant ..... [s^{-1}]

$k_{-1,CM}$ - first order rate constant (canola meal system) ..... [h^{-1}]

k_1 - second order rate constant ..... [$mM^{-1}\ s^{-1}$]

k_1 - first order rate constant (enzyme deactivation) ..... [h^{-1}]

$k_{1,CM}$ - second order rate constant (canola meal system) ..... [mL $mol^{-1}\ h^{-1}$]

k_2 - second order rate constant ..... [$mM^{-1}\ s^{-1}$]

k_2 - first order rate constant (enzyme deactivation) ..... [h^{-1}]

$k_{2,SAE}$ - second order rate constant (canola meal system) ..... [mL $mol^{-1}\ h^{-1}$]

$k_{3,PCM}$ - second order rate constant (canola meal system) ..... [mL $mol^{-1}\ h^{-1}$]

k_3 - first order rate constant (enzyme deactivation) ..... [h^{-1}]

k_4 - second order rate constant ..... [$mM^{-1}\ min^{-1}$]

k_5 - first-order rate constant ..... [min^{-1}]

$k_{5,CM}$ - first order rate constant (canola meal system) ..... [h^{-1}]

k_{ads} - Linear adsorption isotherm coefficient ..... [$mL_{liquid}\ g_{solid}^{-1}$]

k_b - Boltzman constant ..... [$J\ K^{-1}$]

k_d - enzyme decay/deactivation constant ..... [s^{-1}]

k_F - Freundlich isotherm coefficient ..... [$mL_{liquid}\ g_{solid}^{-1}$]

k_H - Henry's constant ..... [Pa]

k_i - arbitrary rate constant ..... [$mM^{1-no}\ s^{-1}$]

k_i^* - arbitrary rate constant at central or average temperature T^* ..... [$mM^{1-no}\ s^{-1}$]

k_La - oxygen mass transfer coefficient ..... [min^{-1}]

$k_L^{CM}a$ - oxygen mass transfer coefficient (canola meal system) ..... [h^{-1}]

k_{L_2} - Langmuir constant ..... [$g_{solute}\ g_{solid}^{-1}$]

k_n - parameter defined by Eq.(B.50) ..... [h^{-1}]

k_p - first-order rate constant for the oxygen utilization by Clark's electrode ..... [min^{-1}]

k_x - first order thermolysis rate constant ..... [min^{-1}]

m - stoichiometric coefficient for the enzymatic reaction ..... [-]

M_{CM} - mass of CM ..... [g]

M_s - total mass of the solid ..... [kg]

$M_{s,i}$ - mass of the solid in the i^{th} fraction of CM ..... [kg]

MU - solid solvent uptake ..... [$mL\ g^{-1}$]

Mw_i - molecular weight of compound i ..... [$kg\ mol^{-1}$]

n - order of the functions Ψ_n ; counter in the summations ..... [-]

$N_{0,i}$ - mass flux from the particles in i^{th} fraction to the fluid ..... [$kg\ s^{-1}$]

n_F - Freundlich constant ..... [-]

no - reaction order ..... [-]

n_{O2} - number of mol of oxygen dissolved in water ..... [-]

P - total pressure in the system ..... [Pa]

P - biomass productivity ..... [$mg\ mL^{-1}\ h^{-1}$]

P_{max} - maximum biomass productivity ..... [$mg\ mL^{-1}\ h^{-1}$]

p - probability level ..... [-]

$p1$ - parameter in Eq.(9.3) ..... [$g\ nkat^{-1}$]

$p3$ - parameter in Eq.(9.3) ..... [-]

pH^{opt} - optimum reaction pH

q_n - roots of Eq.(9.25) ..... [-]

R - universal gas constant ..... [J mol^{-1} K^{-1}]

R_i - radius of the particle in the i^{th} fraction ..... [m]

R_{avg} - average radius of CM particles ..... [m]

r_1 - parameter in Eq.(9.4) and Eq.(B.30) ..... [h^{-1}]

r_2 - parameter in Eq.(9.4) and Eq.(B.30) ..... [h^{-1}]

r_3 - parameter in Eq.(9.4) and Eq.(B.30) ..... [h^{-1}]

r_i - intraparticle radial distance for i^{th} fraction ..... [m]

r_{O2} - rate of change in the oxygen concentration in the CM system ..... [mol h^{-1}]

r_{PCM} - rate of change in the P_{CM} concentration in the CM system ..... [mol h^{-1}]

r_{SAE} - rate of enzymatic transformation of SAE extracted from CM ..... [mol h^{-1}]

S - initial glucose concentration ..... [% w/v]

$S1_{\infty}$ - summation of terms n^{-2} from 1 to ∞ ..... [-]

$S1_{n_0}$ - summation of terms n^{-2} from 1 to n_0 ..... [-]

$S1_{n_f}$ - summation of terms n^{-2} from 1 to n_f ..... [-]

$S2_{\infty}$ - summation of terms n^{-4} from 1 to ∞ ..... [-]

$S2_{n_0}$ - summation of terms n^{-4} from 1 to n_0 ..... [-]

T - temperature ..... [K]

T^* - central or average temperature ..... [K]

t - time ..... [s]

t_r - reaction time ..... [s]

V_g - volume of the gas phase (canola meal system) ..... [m^3]

V_l - volume of the bulk liquid (canola meal system) ..... [m^3]

V_{max} - maximum velocity (activity) ..... [nkat]

V_{max}^{app} - apparent maximum velocity (activity) ..... [nkat]

V_{max}^{opt} - maximum activity at optimum pH ..... [nkat]

V_p - volume of the pores ..... [m^3]

V_{sys} - volume of the reacting solution ..... [L]

v - initial reaction rate/activity/velocity ..... [nkat]

v_{enz} - volume of enzyme added ..... [mL]

v_{pH}^{opt} - initial reaction rate at optimum pH ..... [nkat]

$w0_i$ - initial concentration of the solute in the solid ..... [g_{solute} g_{solid}^{-1}]

$w1_i$ - surface concentration ..... [g_{solute} g_{solid}^{-1}]

w_i - apparent concentration of the solute in the solid ..... [g_{solute} g_{solid}^{-1}]

$\overline{w_i}$ - average concentration of the solute in the solid ..... [$g_{solute}\ g_{solid}^{-1}$]

w_i^* - concentration in the solid for a constant boundary condition ..... [$g_{solute}\ g_{solid}^{-1}$]

w_i^e - the equilibrium concentration of the solute in the liquid ..... [$g_{solute}\ g_{solvent}^{-1}$]

$X_{O_2}^{gas}$ - mol fraction of oxygen in the gas phase (canola meal system) ..... [-]

x_1 - parameter in Eq.(8.1) ..... [-]

x_i - mass fraction of CM particles with radius R_i ..... [-]

Y_c - corrected initial reaction rate ..... [nkat]

GREEK SYMBOLS

α - ratio of the volume of the bulk liquid over the volume of CM ..... [-]

β - proportionality constant ..... [$mmol_{enz}\ mL_{enz}^{-1}$]

γ - parameter characterizing experimental system ..... [-]

Θ - degree of system saturation with the enzyme ..... [-]

η - efficiency ..... [%]

λ - integration variable ..... [s]

ε - molar absorptivity ..... [$M^{-1}\ cm^{-1}$]

ε - porosity ..... [-]

λ_1 - parameter in Eq.(8.1) and Eq.(B.3) ..... [h^{-1}]

λ_2 - parameter in Eq.(8.1) and Eq.(B.3) ..... [h^{-1}]

μ_l - dynamic viscosity of bulk liquid ..... [cP]

Ψ_n - functions defined as $\Psi_n(t) = \int_0^t \frac{\partial \phi}{\partial \lambda} e^{(\lambda - t)k_n} d\lambda$

$\phi_i(t)$ - time-dependent surface concentration for the i^{th} fraction..... [$g_{solute}\ g_{solid}^{-1}$]

ρ_l - apparent density of bulk liquid ..... [$kg\ m^{-3}$]

ρ_s - density of canola meal ..... [$kg\ m^{-3}$]

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Chapter 1

Introduction

1.1 Motivation

A demand from modern society for high quality food products has forced food-related industries to search for technologies that would result in better quality products. Therefore, the introduction of new and the improvement of existing methods for the processing of food material are the ultimate goals for food-related industries.

Among many agricultural commodities in world trade, oilseeds and their products are the most valuable [Shahidi, 1990]. The production of oilseeds in Canada accounts for 3.0% of the world global production, placing Canada in seventh place among worldwide producers. Yet, Canada is the largest producer and exporter of genetically improved types of rapeseeds, known as canola [Shahidi, 1990; Bell, 1993]. These seeds are mainly used as a source of high quality canola oil which accounts for 70.0% of all edible oils produced nationwide. A solid residue, called canola meal (CM), remaining after deoiling process of the seeds is used as a source of nutrients for livestock and poultry [Bell, 1993; Shahidi, 1990, Clandinin *et al.*, 1986]. Furthermore, as a protein-rich commodity, CM has a potential to replace soybean products as a major source of high quality proteins for human consumption. During the 1980's, an average of 900,000 tonnes per year of CM was produced. An increasing trend has been observed in the present decade, and an average yearly production of CM was 1,200,000 metric tonnes between 1990-95. The 1995-96 production was even higher at 1,724,000 tonnes per year [Statistics Canada, 1996]. Unfortunately, high levels

of glucosinolates, fiber, phytate and phenolics prevent a replacement of well-recognized soybean products by these of CM origin.

The level of phenolic compounds in CM is at least an order of magnitude higher than in soybean meal [Kozlowska *et al.*, 1990; Zadernowski and Kozlowska, 1983]. Although not directly harmful to humans, phenolics are believed to be responsible for the changes in the internal organs of livestock and poultry when meals are included as part of their diet [Butler *et al.*, 1982]. Their presence is also related to the bitter flavour and dark colour of the meal and the CM protein concentrates [Clandinin, 1961; Goh *et al.*, 1979; Butler *et al.*, 1982].

Among the reported methods for the removal of phenolics from rapeseeds, not one is completely successful. Some of these processes decrease the phenolic content of the meal by 90.0%, but they are not economically feasible. Furthermore, a decrease in the nutritional value of the treated meals has raised some concerns about the utilization of these methods. Consequently a need for new and better technologies for upgrading CM is still an important issue.

One of the possible solutions is a process based on enzymatic treatment. It would require mild reaction conditions, what in turn can considerably reduce the operating cost of a process and can decrease the risk of degradation of valuable food components. In this respect, the enzymatic process for decreasing the meal's phenolic content may prove to be better than the existing methods.

1.2 Problem statement

This research project is focused on the development and evaluation of a new process, based on the enzymatic reaction, for decreasing of the phenolic content in CM. Since CM can be considered as part of the human food chain, many issues have to be addressed before such a process can be commercially applied. The interactions between products of the enzymatic reaction and CM have to be examined. The effect of operating conditions on the quality of the resulting meal also must be taken into consideration since a trade-off between optimum process conditions and an overall quality of the processed meal can be expected.

1.2.1 Objectives

The objectives of this work are: (1) to develop a new enzymatic method to decrease the phenolic content in commercial canola meal, and to compare the new method with existing processes for the reduction of meal's phenolic content; (2) to perform an introductory evaluation of the quality of the treated meal; (3) to produce the new enzyme system from white-rot fungi *Trametes versicolor* ATCC 42530, and to characterize it using the phenolic compounds found in CM as substrates; (4) to determine the mechanism of the investigated enzymatic transformations, and to describe it by mechanistic and/or semi-mechanistic mathematical models; (5) to separate products of the enzymatic transformation of the main phenolics found in canola meal, and to elucidate their structures in order to determine their effect on the quality of the treated meal; (6) to formulate a mechanistic mathematical model describing the new enzymatic process for the decrease of phenolic content in a commercial canola meal.

1.2.2 Expected contributions

The following contributions in the field of applied sciences, including chemical and biochemical engineering, chemistry, applied enzymology, and mathematical modeling are expected:

- A robust method for the production of the polyphenol oxidase system from *Trametes versicolor* will be proposed.
- A new polyphenol oxidase system from *Trametes versicolor* will be characterized.
- Products from the enzymatic transformations of sinapic acid and sinapine will be separated and identified. This should bring new information about biodegradation of lignin by the white rot fungi.
- Enzymatic reaction pathways involving polyphenol oxidase and sinapic acid will be elucidated.
- Mathematical model simultaneously describing effects of substrate or substrates concentrations, enzyme concentrations, pH and temperature will be presented, and recommendations based on the model prediction regarding the optimum condition for testing oxygen-utilizing enzymes will be given.

- The possibility of an enzyme-based process for the decrease of phenolic content in CM will be evaluated. The qualitative and quantitative data from this process will lead to a better understanding of enzymatic processes in complex environments.
- A mathematical model describing the new process will be formulated, and values of the model parameters will be estimated using experimental data. Some theoretical analysis of the process will be carried out.
- A novel approach for solving a system of ordinary differential equations and a partial differential equation describing extraction of a solute from a spherical matrix into a finite volume solution, followed by a chemical reaction, will be evaluated.

1.2.3 Organization of the thesis

The thesis is divided into five parts. In the first part the literature review providing the insights for this project is presented. The second part offers the reasoning for the experimental and theoretical approach chosen in this work, and the description of the experimental systems applied in this study, with additional information concerning analytical techniques used for analysis. In the third part the results obtained are presented and discussed. The fourth part consists of the conclusions and recommendations for future research. The fifth part is composed of several appendices, which contain additional results as well as some theoretical aspects of the presented work.

Part I

Literature Review

Chapter 2

Rapeseed and Canola

In order of importance, the main oilseed crops produced in Canada are rapeseed/canola, soybean, flaxseed, sunflower and mustard seeds. Although rapeseeds have been used for over 3000 years, their growth on a commercial scale in developed countries only started during World War II. At that time, the superb lubricating qualities of rapeseed oil were recognized. However, the use of this oil as a source of edible vegetable oil was limited because of some concerns arising from its composition, mainly erucic acid. In the mid-1970's, genetically improved varieties of rapeseed were introduced into the market and these crops became a main source of edible oil in Canada. These new types of rapeseeds, known as canola, contain less than 2.0% erucic acid in the oil and no more than 30μg of goitrogenic glucosinolates per gram of meal. As a result, the US Food and Drug Administration granted Canola GRAS (Generally Recognize As Safe) status [Shahidi, 1990].

2.1 Canola Meal

Canola meal (CM) is a byproduct of the deoiling process of canola seeds. The Canadian production of CM in 1995-96 was 1,724,000 metric tonnes [Statistics Canada, 1996]. The production of the meal is expected to increase further due to the nutritional values of the meal and relatively low cost comparing to other types of oil crop meals. The price of CM is approximately 20.0-30.0% lower than that of soybean meal containing 44.0% protein [Clandinin *et al.*, 1986; Statistics Canada, 1996]. It is mainly used in rations for livestock and poultry diets. However, a more widespread use of CM is limited due to a high level of toxic substances such as phytate,

glucosinolates and phenolics. Consequently, if economically feasible processes for the reduction of toxic components in CM were developed, canola could potentially become a major source of protein in the production of human food ingredients [McCurdy, 1990]. Moreover, it has been suggested that such processes might be essential for the future of the canola meal industry, in order to compete with products based on soybean protein [Tzeng *et al.*, 1990].

2.1.1 Composition of canola meal

Canola meal accounts for approximately 60.0% of the seed weight [Shahidi, 1990]. Its general composition is presented in Figure 2-1. CM contains 35.0-40.0% protein, and 27.0-30.0% hulls. The rest of the meal is composed of carbohydrates (13.0%), glucosinolates (0.5%), phenolics (0.5-3.5%), phytate (2.0-5.0%), fiber (11.0%), minerals, and vitamins [Shahidi, 1990; Clandinin *et al.*, 1986]. Commercially available meal usually contains up to 3.0% residual oil, and approximately 8.0% moisture.

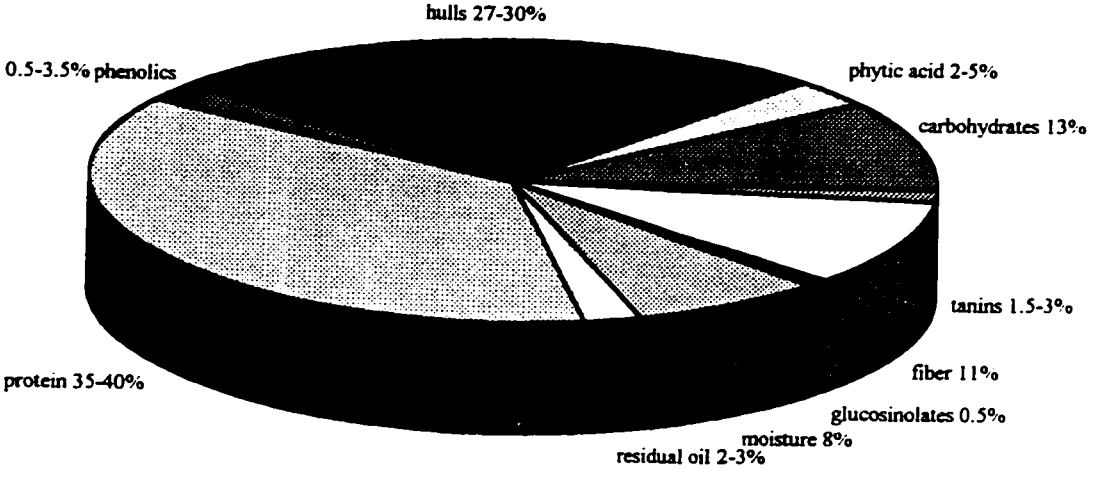


Figure 2-1. Composition of canola meal.

The most valuable components of CM are the proteins, which have a comparable amino acid composition to that of soybean proteins [Ohlson and Anjou, 1979; Sarwar *et al.*, 1984]. Moreover, CM contains more threonine and tryptophan [Diem and Lenter, 1975; Larsen and Sorensen, 1985], and more sulfur containing amino acids (methionine and cysteine).

The anti-nutritional properties of CM are associated with its fiber content and high levels of glucosinolates, phytate and phenolics [Clandinin *et al.*, 1986]. The main concerns related to the presence of these compounds are: a reduced metabolizable energy of the meal caused by the non-digestible fiber [Clandinin *et al.*, 1986]; reduced bioavailability of multivalent metal ions due to phytate [Serraino and Thompson, 1984; Bell, 1993]; and possible formation of a potentially dangerous isothyocyanates during the hydrolysis of glucosinolates [Sosulski *et al.*, 1972; Shahidi, 1990]. The effects related to the presence of phenolic compounds in CM will be described in more detail later in the text.

2.2 Food phenolics

Ho [1992] described 'phenolics' or 'polyphenols': "... as a substance which possesses an aromatic ring bearing one or more hydroxy substituents, including functional derivatives (esters, methyl ethers, glucosides, etc.)". These kinds of compounds occur commonly in food material and can be classified into three groups; simple phenols and phenolic acids, hydroxycinnamic acid derivatives, and flavonoids [Ho, 1992]. Phenolics may contribute directly to the desirable and undesirable aromas and tastes of food. Therefore, they are closely associated with the sensory and anti-nutritional quality of fresh and processed plant food [Ho, 1992]. On the other hand, the healing properties of herbal plants are related to the presence of flavonoids. The physical and pharmacological properties of plant polyphenols are listed in Table 2-1 .

2.2.1 Phenolics in canola meal

The phenolic content of CM is relatively the same as in other types of rapeseed meals, therefore the name rapeseed/canola can be used when describing the effects of these compounds. The content of phenolics in rapeseed/canola flour is much higher than in other oleaginous seeds (Table 2-2).

The phenolics level in CM is 10.0 to 30.0 times higher than found in soybeans [Zadernowski and Kozlowska, 1983; Dabrowski and Sosulski, 1984b; Kozlowska *et al.*, 1990]. This amount varies with the type of cultivar, as well as location of growth [Mueller *et al.*, 1978a]. Phenolic compounds occur in CM as flavonoids (tannins), lignin, and phenolic acids and/or their

Table 2-1. Plant polyphenols: physiological and pharmacological properties [Haslam, 1989].

1.	*Foodstuffs and beverages* (a) Flavor, palatability and acceptability (astringency) (b) Nutritional value (c) Non-biological hazes, ageing of wines (d) Herbal teas - oesophageal cancer (e) Toxic effects - molluscicidal action
2.	*Herbal medicines* (a) Effects on vascular system - blood pressure and capillary action (b) Antiviral (antiherpetic) effects (c) stomach disorder, dysentery, diarrhoea, haemorrhages (d) Dressing of burns and inflammations (e) Inhibition of direct mutagens (f) Microbial toxicity - antiseptic action
3.	*Chemical defense in plants*

Table 2-2. Total content of phenolic acids in some oilseed flours[a]

Flour	Total phenolics (mg/100 g)
Soybean	23.4
Cottonseed	56.7
Peanut	63.6
Rapeseed	639.9

[a] according to Kozlowska and Zadernowski [1988]

derivatives [Shahidi, 1992; Bibi *et al.*, 1991]. Among them, phenolic acids and their derivatives (esters) are the most common, constituting for up to 3.0% w/w of the meal. They exist in three forms: free acids, soluble phenolic acid esters, and insoluble-bound phenolic acids esters [Sosulski *et al.*, 1980; Krygier *et al.*, 1982a; Shahidi and Naczk, 1992].

The total amount of free phenolic acids that are present in CM is not greater than 10.0% of the total phenolics content of the meal [Krygier *et al.*, 1982b; Shahidi and Naczk, 1992]. Regardless of the variety of meal, the most abundant acid is sinapic acid (SA), 3,5-dimethoxy-4-hydroxy cinnamic acid (Figure 2-2). It accounts for 99.0% of all free acids found in the meals [Krygier *et al.*, 1982b; Shahidi and Naczk, 1992]. Other acids that are found in this fraction include benzoic acids (p-hydroxybenzoic, vanillic, syringic and gallic) and cinnamic acids (coumaric, caffeic, ferulic) (Figure 2-2). The insoluble-bound phenolic acids esters constitute a

minor portion of the total phenolics, usually below 0.1 % [Shahidi, 1992; Krygier *et al.*, 1982a; Kozlowska *et al.*, 1983, Shahidi and Naczk, 1992].

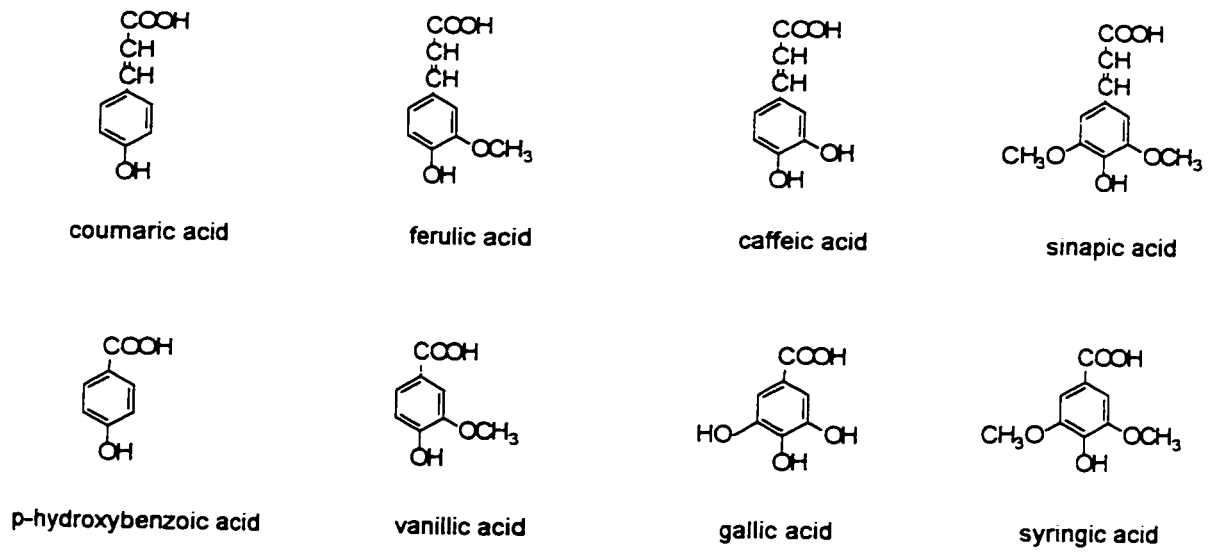
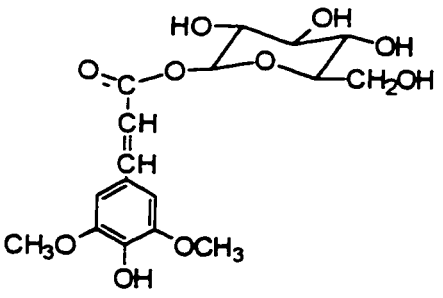
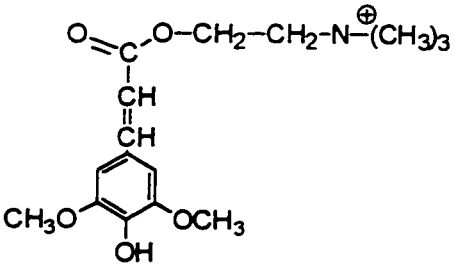


Figure 2-2. Chemical structures of basic phenolic acids of canola meal.

The SA esters (SAE) comprise of 90.0-95.0% of the total phenolics content of rapeseed/canola meals. Fenton *et al.* [1980] showed that at least eight of these esters can be isolated from the methanol extract of CM. The most abundant among them is sinapine (SIN), the choline ester of SA [Clandinin, 1961; Krygier *et al.*, 1982b; Mueller *et al.*, 1978a, Bouchereau *et al.*, 1991] (Figure 2-3). It accounts for 57.0-76.0% of all SAE present in the meals. The level of SIN varies depending on cultivar, ranging from 0.92% in so called Polish rapeseed [Austin and Wolff, 1968] to 1.12-2.26% in the present canola/rapeseed varieties [Mueller *et al.*, 1978a; Fenwick *et al.*, 1984]. A higher content of SIN was found in *Brassica napus* rapeseed cultivars than in *Brassica campestris* cultivars [Mueller *et al.*, 1978a]. Blair and Reichter [1984] reported a higher content of SIN (2.67 and 2.85%) in the defatted cotyledons, but those results included free SA, as well as other compounds. One of them could have been 1-*O*-β-D glucopyranosyl sinapate (Figure 2-3), which can be found in the ethanolic extracts of CM [Wanasundara *et al.*, 1994; Amarowicz and Shahidi, 1994].



glucopyranosyl sinapate



sinapine

Figure 2-3. Chemical structure of sinapine and glucopyranosyl sinapate.

Tannins occur in CM in hydrolyzable and/or condensed form [Shahidi, 1992]. Mitaru *et al.* [1982] reported that canola and rapeseed hulls contained 0.02-0.22% extractable tannins. Blair and Richter [1984] showed that defatted rapeseed and canola cotyledons contained 0.09-0.39% and 0.23-0.54% tannins, respectively. Higher values (0.68-0.72%) of condensed tannins were reported by Shahidi and Naczk [1989]. On the other hand, Leung *et al.* [1979] reported that no tannins were detectable in rapeseed cotyledons, but hulls contained 0.1% condensed tannins. The structures of basic units of condensed tannins of CM are shown in Figure 2-4.

2.2.2 Effect of phenolics on the quality of canola meal

The anti-nutritional effects of phenolic acids and flavonoids are related to their strong binding affinity to dietary proteins, making them partially unavailable for digestion [Shahidi, 1992]. Furthermore, the process of phenolic binding to a protein molecule is related to the phenomenon of astringency [Haslam, 1989] which is directly responsible for the taste of protein.

Phenolic compounds are multidentate ligands that are able to bind simultaneously (*via* different phenolic groups) at more than one point on the protein surface [McMannus *et al.*, 1981; Gustafson and Holm, 1952]. Hagerman and Butler [1981] also showed that the affinity of a given polyphenol can be different for different proteins. The interactions of polyphenols and proteins can be either reversible or irreversible [Haslam, 1989]. The reversible binding can become irreversible when polyphenols and proteins are brought into contact under specific conditions which favor phenolics oxidation (*e.g.*, alkaline pH, O_2). Polyphenols may then be oxidized to quinones and

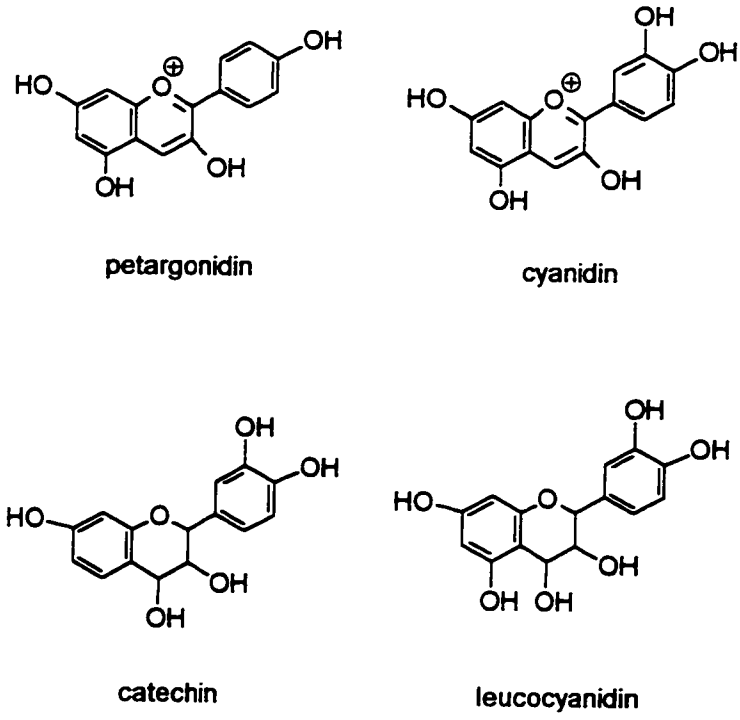


Figure 2-4. Structures of basic units of condensed tannins.

form a covalent linkage with proteins, making the association irreversible [Kumar and Singh, 1984; Haslam, 1989, Shahidi, 1992]. The irreversible complexation reduces the quality of the proteins.

Phenolics that exist in rapeseed/canola meals are responsible for the dark colour, high astringency and bitterness of these commodities. SIN is directly responsible for the bitter taste and unpleasant flavour of the meal [Sosulski *et al.*, 1976]. It has been shown [Ismail *et al.*, 1981] that the bitterness of SIN is 20.0% higher than that of SA. On the other hand the magnitude of astringency for these two compounds exhibits an opposite pattern [Ismail *et al.*, 1981]. Since the definition of a compound's astringency is based on the compound's affinity towards protein, a higher astringency for SA would indicate that the binding of SIN with protein is suppressed by the presence of a choline functional group in the SIN molecule. This implies that methods for the decrease of SIN content in the meal, that are based on the hydrolysis of SIN to SA and choline, may not be suitable for the production of protein concentrate, even though the bitterness of such a product would be lower.

The main problem caused by the high level of SIN in CM is the production of 'tainted' brown shell eggs [Hobson-Frohock *et al.*, 1977; Clandinin *et al.*, 1977; Mueller *et al.*, 1978b]. The fishy odor of these eggs is related to the presence of trimethylamine (TMA) [Hobson-Frohock *et al.*, 1973; Mueller *et al.*, 1978b], which is produced from choline [Clandinin, 1961]. Normally, the TMA is oxidized to water soluble trimethylamine oxide by the action of an oxidizing enzyme, TMA oxidase [Uppstrom and Johanson, 1985; Hobson-Frohock *et al.*, 1973]. This reaction is, however, suppressed in the presence of SIN, which shows the inhibitory effect on TMA oxidase [Butler *et al.*, 1982]. It has also been reported [Butler *et al.*, 1982; Fenwick *et al.*, 1981; Fenwick *et al.*, 1984] that tannins are partially responsible for the tainting of brown shell eggs, because of their inhibitory effect on TMA oxidase. Since TMA is organoleptically detectable when its level exceeds 0.8 to 1.0 ppm [Fenwick *et al.*, 1979; Goh *et al.*, 1979], the rations for poultry can contain no more than 0.1% SIN [Goh *et al.*, 1979]. Consequently the amount of CM included in poultry diets should not exceed 5.0%, even though the use of CM is highly recommended [Clandinin *et al.*, 1986]. The inverse effect of rapeseed/canola tannins on the quality of a feed stuff is related to a decrease in bioavailablity of meal's proteins. Tannins bind with proteins reducing their digestibility. Furthermore, if the tannin content is higher than 20.0 mg/g of dry matter the rejection of feed by grazing animals has been observed [Donnely and Anthony, 1969]. It is also suspected that the high level of polyphenols is the cause of thyroid enlargement and skeleton deficiencies when rapeseed meals are a major part of a diet [Butler *et al.*, 1982]. However, in a review on the toxic effects of naturally occurring polyphenols, Singelton [1981] pointed out that tannins are only directly harmful to humans in uncommon and usually predictable circumstances, but they can be toxic to other organisms, such as molluscoidal, therefore affecting the human food chain.

2.2.3 Measurements of the phenolic content in canola meal

Up to date, there is no single method recommended to use for a determination of phenolics content in canola meals, and as pointed out by Shahidi and Naczk [1992] developing a standardized method for analysis and quantitation of these compounds is warranted.

The existing methods that are used to measure the phenolics content in the rapeseed/canola meals require extraction of these compounds with organic solvents, followed by their quantitation

either by a direct spectrophotometric (DS) [Kolovrat 1990; Naczk *et al.* 1992; Pink *et al.* 1994; Blair and Richter 1984], or by a chemical spectrophotometric (ChS) [Mueller *et al.*, 1978a; Naczk *et al.*, 1986; Tzagoloff, 1963a; Austin and Wollf, 1968; Ismail and Eskin, 1979] methods, or by methods that are based on the chromatographic separation techniques such as thin layer chromatography (TLC) [Fenwick, 1979; Uppstrom and Johnsson 1985], gas-liquid chromatography (GLC) [Krygier *et al.*, 1982a; Dabrowski and Sosulski, 1984a; Kozlowska *et al.* 1983], or high performance liquid chromatography (HPLC) [Clausen *et al.*, 1983; Bouchereau *et al.*, 1991; Bjerg *et al.*, 1984; Henning 1982]. The spectrophotometric methods are faster than the separation techniques, but are nonspecific, which in some case can be considered as a disadvantage. For example, the DS method measures phenolic content in the meal in terms of all SAE plus free SA, whereas the ChS method measures only all SAE. Recently it has also been shown that results obtained with the ChS method also include free sinapic acid [Łącki and Duvnjak, 1996b]. On the other hand, the separation techniques give a detailed composition of the analyzed phenolics. The choice of the analytical method used to measure the phenolic content in the rapeseed meals depends, therefore, on individual preferences and/or on the goals of a particular research project.

The extraction of phenolics from the meal is usually carried out with methanol or in some cases with an acetone-water mixture [Blair and Richter, 1984; Krygier *et al.*, 1982a]. The reported extraction conditions varied in terms of a temperature, extraction time, meal to solvent ratio and number of consecutive extraction. On average, a triplicate extraction with the overall ratio of meal to extracting solvent of one to 100-150 can be considered sufficient to extract all of the free phenolic acids and all of their esters. On the other hand, the efficiencies of tannin extraction are dependent on the type of the organic solvent, presence of water, number of extraction steps and solvent to meal ratio [Naczk and Shahidi, 1991].

2.2.4 Removal of phenolics from rapeseed/canola

The possibility of growing selective varieties of rapeseed/canola with zero, or almost zero SIN content seems to be the best solution to the problems associated with its presence. However, an extensive screening of almost 200 types of CM failed to show one type without SIN [Vogt *et*

al., 1993; Boucherean *et al.*, 1991]. Although recent advances in genetic engineering indicate that there are possibilities for the development of zero SIN rapeseed/canola varieties [Vogt *et al.*, 1993], the problem of high levels of other phenolics in CM would still exist. Consequently, technologies that could decrease the phenolics content in the rapeseed/canola meals are still needed.

Processes for the reduction of the phenolics content of CM have been reported, but none of them are fully successful. These methods were either designed exactly for the purpose of decreasing the phenolics content, or were reported as an additional effect of other processes focused on the reduction of meal glucosinolates. Generally, the efficiencies of these methods, expressed in terms of phenolic content decrease, are not greater than 90.0% [Van Etten *et al.*, 1969; Goh *et al.*, 1982; Srivastava and Hill, 1976; Fenwick *et al.*, 1979; Adu-Peash *et al.*, 1989, Naczk and Shahidi, 1989; Naczk *et al.*, 1992; Naczk *et al.*, 1986; McCurdy and March, 1992]. The reported data also indicated that the results obtained with various methods were very strongly dependent upon the meal type and can vary by up to 43.0% [Goh *et al.*, 1982].

For the purpose of this review, the following classification of existing methods for the decrease of the phenolic content in CM (or rapeseed) is proposed:

- methods based on extraction
- methods based on extraction with chemical reaction
- methods based on chemical reaction.

The first group contains processes in which the decrease of phenolics content was a result of pure extraction. Processes in which the decrease is the sum of individual effects of the extraction and the chemical reaction steps fall into the second category, whereas processes in which the decrease in phenolics content is only caused by a some sort of chemical reaction belong to the last group. Since the extraction step also exists in most of the processes that can be classified into the third group, the amount of extractant and the meal extractant uptake (Mu_i, i stands for the extractant type) must be considered to differentiate between the second and the third group of processes. If Mu_i is bigger than the amount of extractant used then a process would fall into the chemical reaction category since extraction is only responsible for the transport of phenolics to the reaction site. Therefore, if the reaction does not take place, the extracted phenolics would remain entrapped in the meal owing to the extractant uptake and, consequently, their concentration in the

meal would not change. If, on the other hand, the volume of the extractant is greater than MU, then the process falls into the second category. The use of extractant uptake as the distinction parameter would allow processes based on the same chemical reaction to be placed into either third or second group of the proposed classification, depending on the conditions of particular treatment.

A list of reported methods arranged according to the presented classification, with a brief description of principle of each process and its efficiency, is given in Table 2-3.

Most of the extraction methods employed different types of organic solvents [Krygier *et al.*, 1982a; Kozlowska *et al.*, 1975; Naczk *et al.*, 1992; Dabrowski and Siemianiak, 1987; Bibi *et al.*, 1991; Bouchereau *et al.*, 1992]]. These methods are examples of analytical techniques used for the extraction of phenolics from the meal prior to their quantitative determination and therefore, only few of them are cited in Table 2-3. Yet, Dabrowski and Siemianiak [1987] showed that a laboratory multistage counter-current extraction with solvents mixture, composed of acetone, methanol and water, resulted in a 97.0% decrease in SIN content of the rapeseed meal. The extraction processes can, however, have negative effects on the overall quality of treated CM due to the presence of traces of harmful organic solvents in the processed meal. An alternative method of extraction uses an aqueous medium. However, this method is not widely used, probably due to a possible extraction of valuable meal components. One of the reported methods resulted in 20.0% loss of dry matter of a treated CM, however its effectiveness for phenolics decrease was not specified [Lo and Hill, 1972]. On the other hand, McCurdy and March [1992] showed that 1 hour extraction of the commercial meal at a meal to water ratio of 1:9 carried out at room temperature resulted in a 40.0-60.0% decrease in phenolic content. Taylor *et al.*, [1993] also used water extraction to determine the rates of sinapine leakage from *Brassica* seeds. According to these results a 24 hour-long soaking of the seeds at the seed to water ratio of 1:1000 did not cause a complete extraction of sinapine from the seeds. The latter method is similar to the so called "diffusion extraction" used to reduce glucosinolate content of canola seeds [Sosulski *et al.*, 1972].

The addition of a reaction step to the process should decrease the amount of extractant required due to a constant presence of the concentration gradient, the driving force for any extraction. The use of a lower amount of the extractant would have a beneficiary effect on the meal quality, because a smaller amount of the meals' valuable compounds would be extracted.

Table 2-3. List of the processes used to decrease the phenolics content in canola meal and their efficiencies (η)

Treatment	Extractant	R	η (%)	Comments	Ref.
Extraction					
Duplicate extraction for 1 h each at room temperature	1% HCl in MeOH	20.0	100.0	analytical method	Bibi *et al.*, 1991
Quadruplicate extraction for 20 min each at 80°C	MeOH	20.0	100.0	analytical method	Bouchereau *et al.*, 1992
Triplicate soaking of the seeds, each time for 12 hours	Citric acid	1.0	61.0		Schwenke *et al.*, 1990
Bed extraction of the meal for two hours at the flow rate of 1.0 L/h, at room temperature	EtOH	2.0	18.0 67.0		Goh *et al.*, 1982
Batch extraction for 1.5 and 0.5 h at room temperature	EtOH	8.5	90.0	pilot scale	McCurdy and March, 1992
Crushed seeds extracted using Polytron at room temperature	Hexane-MeOH-H_2O	6.7	76.0	95% w/v MeOH, 5% H_2O	Diosady *et al.*, 1985
Batch extraction for 1 hour at room temperature	H_2O	9.0	40.0 60.0	pilot scale	McCurdy and March, 1992
Soaking of the seeds at room temperature for 24 h	H_2O	1000	<100.0		Taylor *et al.*, 1993
Extraction with Chemical Reaction					
Seeds extracted with extractant during the crushing step	Hexane-MeOH-NH_3	6.7	73.0	with or without 5.0% of H_2O	Naczk *et al.*, 1986
			67.0		Diosady *et al.*, 1985
		2.5	59.0	pilot scale	
		3.3	61.0	pilot scale	Diosady *et al.*, 1987
		6.7	78.0	pilot scale	
		6.7	69.0 78.0		Naczk and Shahidi, 1989
			75.0 89.0		Naczk *et al.*, 1992
Triplicate soaking of the seeds, each time for 12 hours at pH 9.0	$(NH_4)_2CO_3$	1.0	84.0		Schwenke *et al.*, 1990
Bed extraction of the meal for 2 hours at the flow rate of 1 L/h	EtOH-NH_3	2.0	42.0 90.0	1.0 M NH_3	Goh *et al.*, 1982
Batch extraction at room temperature for 0.5 h	NH_3-H_2O-MeOH	10.0	69.0	10% NH_3, 5% H_2O, 85% MeOH	McCurdy and March, 1992

Table 2-3. Continued

Treatment	Extractant	R	η (%)	Comments	Ref.
Chemical Reaction					
Autoclaving for 1 hour at atmospheric pressure	H_2O	na	42.0		Van Etten *et al.*, 1969
			64.0		Fenwick *et al.*, 1979
Steaming: 50g of steam per 1 kg of meal	H_2O	0.05	7.0		Srivastava and Hill, 1976
			42.0		Fenwick *et al.*, 1979
Ammoniation	H_2O		74.0		McGregor *et al.*, 1983
			90.0		Kirk *et al.*, 1966
		0.2	36.0		Fenwick *et al.*, 1979
Meal mixed with $Ca(OH)_2$ at ratio of 24:1 for 12 h	H_2O	0.08	36.0	mixture was occasionally mixed	Fenwick *et al.*, 1979
		0.16	84.0		
		0.54	96.0		

As shown in Table 2-3, all of the methods that involve chemical reaction are based on alkali treatments. Austin and Wolff [1968] showed that under alkaline conditions SIN is readily hydrolyzed to SA and choline. Although methods based on this principle are suitable to solve problems related to the production of defected eggs (decrease in SIN content), they cannot be used in the processes for preparing protein concentrates because of already discussed effects of SA and choline on the protein's quality. Although it was shown [Schwenke and Dabrowski, 1990] that the interaction of SA with canola meal protein is an example of reversible binding, it becomes irreversible under alkaline conditions. Rubino *et al.* [1995] showed that SA is converted under alkaline conditions to thomasidioic acid, a lignan-type compound. The occurrence of irreversible protein- polyphenol interactions at high pH [Haslam, 1989] causes some concerns related to protein quality. Furthermore, the use of ammonia in the treatment of rapeseed/canola resulted in palatability problems when treated meals were included in cattle diets [Mustakas *et al.*, 1968]. Treatment of the meal with MeOH-ammonia-hexane causes a decrease in canola protein whipping capacities [Naczk *et al.*, 1986]. Another method employing alkaline hydrolysis is based on a treatment of the meal with calcium hydroxide [Fenwick *et al.*, 1979]. The efficiency of this method

strongly depends on the meal moisture content, the higher the moisture the lower residual SIN content. At a moisture level of 35.0%, the level of SIN was reduced by 96.0%. One of the patented processes for the decrease of glucosinolates content is also based on lime treatment [Unilever, 1977]. However, the lime treatment may decrease the quality of the meal's proteins, due to the change in the character of protein-phenolics interactions from reversible to irreversible binding. Another group of processes based on a chemical reaction consists of methods employing a temperature-governed hydrolysis or autolysis of meal phenolics. Fenwick *et al.* [1979] reported that using those methods, a 64.0% decrease of SIN content was observed. Since water was removed either by lyophilization or air-drying, this decrease was a result of chemical reaction only. The products of this reaction were not reported and, therefore, an evaluation of this method is rather difficult. However, one could not assume that this kind of treatment would not have negative effects on meal proteins.

For the time being there is no industrial treatment focused on the removal of phenolics from the meal during its industrial processing. A study done on the composition of the meal after different stages of the meal production in a meal processing plant showed that SIN content decreased by 25.0% only after the meal toasting step [Dabrowski *et al.*, 1989]. On the other hand, an industrial processing of rapeseed seeds seems to alter physical properties of the meals, as compared to the properties of the meals prepared in laboratory. Difficulties in extracting phenolics from commercial canola meals, using organic solvents, were reported [Naczk *et al.*, 1986; Goh *et al.*, 1982]. In these cases the extraction processes were only 30.0% effective. The explanation for this phenomenon was not given. One possible reason might be the formation of non-permeable structures of proteins during the industrial extraction of canola oil [Unger, 1990]. In such a case, the solvent could not penetrate through the protein agglomerates, and the phenolics entrapped within would not be extracted. This hypothesis is supported by a higher extractibilty of phenolics by solvents supplemented with water [Naczk *et al.*, 1986; Krygier *et al.*, 1982b].

Chapter 3

Enzymatic processes

Over the past few decades, the number of industrial applications using enzymes has drastically increased. One of the reasons for this is a requirement for mild reaction conditions, which would reduce the operating cost of a process considerably and decrease the risk of degradation of valuable food components.

Commercially produced enzymes can originate from three different natural sources: plant, animal and microbial [Crueger and Crueger, 1984]. Some enzymes can also be synthesized, but large scale chemical synthesis is presently impractical, if not impossible [Lee, 1991]. Since the doubling time of microbes is much smaller than that of plant or animals, microbial production most efficiently fulfills industrial demands [Bailey and Ollis, 1986]. Furthermore, many useful industrial enzyme preparations are not highly purified, being mixtures of enzymes with different catalytic functions. In some cases, the use of these preparations (several enzymes) can be more efficient than sequential catalysis by a separated series of enzymatic treatments [Bailey and Ollis, 1986].

3.1 Enzymatic treatments of canola meal

The use of enzymatic treatment as part of the processing of rapeseed/canola seeds and meals has already been studied [Sosulski and Sosulski, 1990; Olsen *et al.*, 1987; McCurdy and March, 1992]. Most of these processes were designed to alter the structural properties of the meals or seeds through the solubilization of the polysaccharide material of cell walls. These treatments produced meals with a higher accessibility in terms of further processing, such as oil and protein

extraction. Methods based on this principle were also applied to treat sunflower and palm-kernel meals [Dusterhoft *et al.*, 1993; Dominguez *et al.*, 1995]. The enzymatic upgrading of CM, through the reduction of phytic acid content, was also studied [Nair and Duvnjak, 1992; Al-Asheh and Duvnjak, 1994]. This process, based on solid state fermentation, resulted in 90.0-100.0% removal of phytate. Bearing all of these results in mind, it could be expected that a decrease in the content of phenolic compounds in CM may also be achieved by the means of enzymatic treatment if the proper enzymes are used.

3.1.1 Enzymatic transformations of phenolics

Ideally, the decrease in the phenolics content in CM by enzymatic reactions should be based either on the aromatic ring cleavage or the disappearance of phenolic or quinoic- like structures directly responsible for dietary protein bonding. The benzene ring is a constituent of many chemicals found in nature, therefore, not surprisingly, there are a large number of enzymes that can attack this structure. Most often these enzymes, known as dioxygenases, utilize molecular oxygen to break an aromatic nucleus [Sariaslani, 1989]. However, for such reactions to happen, an aromatic structure has to be modified by the action of other oxidizing enzymes [Higuchi, 1982; Kirk, 1983], such as laccases and polyphenol oxidases (PPO) [Butt, 1980]. As with most oxidizing enzymes, laccases and PPO contain copper as a reducing agent. Very often these two enzymes coexist in a preparation obtained from cultures of a white rot fungi. These enzymes are secreted by the fungi in order to biodegrade lignin [Morohoshi *et al.*, 1987]. The products of this transformation can be then used by the fungi as a nutrient source. However, such a utilization of the lignin can not be accomplished without the break down of an aromatic ring [Ishihara, 1991].

The fission of an aromatic ring by oxygenases requires a presence of hydroxy groups in *ortho-* or *para-* positions [Tai *et al.*, 1983; Ishihara and Nishida, 1983; Higuchi, 1982]. If these groups are missing then the aromatic part of the compound must be changed in order to fulfill the requirements for the ring cleavage. Such a structure alteration can be accomplished by a combined action of at least two types of enzymes, oxidases and reductases. This kind of reaction proceeds through the conversion of substrate free phenolic groups into phenoxy radicals and then into *p*-quinones [Ishihara and Nishida, 1983]. These quinones are further converted into *p*-diphenols by

the action of quinone reducing enzymes. If the methoxy group is attached to the neighboring carbon of the aromatic ring, then a demetylation reaction would produce an *ortho*-diphenol. It was reported that enzymes from white rot fungi can catalyze a transformation of the methoxy group. Reduction of quinones to their respective phenols can be completed by the quinone oxidoreductase action of laccase and cellobiose [Ishihara, 1991], or laccase and glucose reductase [Green, 1977; Szklarz and Leonowicz, 1986]. Such a created *ortho*- or *para*- phenol can than be cleaved by one of the oxygenases.

3.1.2 Polyphenol oxidases of *Trametes versicolor*

It was shown that the cleavage of aromatic nuclei can be catalyzed by the enzyme metapyrocatechase, secreted from the bacterial culture *Pseudomonas* [Kojima *et al.*, 1961; Kawakami, 1976]. Although the presence of metapyrocatechase in cultures of the white rot fungus is rare [Ishihara, 1991], Khan and Overend [1990] reported that an enzyme with similar abilities can be produced by the white rot fungi *Trametes versicolor* ATCC 42530 strain. This enzyme showed properties of an oxygenase, by transforming catechol into muconic acid semialdehyde. It was produced late in the growth cycle, and did not require addition of an inducer, which is neccesary for the production of a peroxidase and laccases that are induced by ferulic acid [Paszczynski and Lobarzewski, 1984; Leonowicz *et al.*, 1978] and xylidine [Rogalski *et al.*, 1990; Fåhraeus and Reinhammar, 1967], respectively.

Since SA and its derivatives have only one hydroxy group attached to the aromatic ring, therefore in order to cleave this ring, a modification resulting in an *ortho*- or *para*-hydroxy compound is required. The formation of quinones from benzoic and cinnamic acid derivatives, the main constituents of CM phenolics, by the laccase of white rot fungus has been reported [Lundquist and Kristersson, 1985; Ishihara and Ishihara, 1976]. The transformation of syringic acid (3,5-dimethoxy-4-hydroxy benzoic acid) by the laccase of *T. versicolor* to 2,6-dimethoxy-*p*-benzoquinone has also been shown [Leonowicz *et al.*, 1984]. Since syringic acid and SA have the same aromatic part of the structure, a similar product from a SA transformation by this enzyme could be expected. Such a compound could be cleaved either by the new oxygenase system or by the metapyrocatechase.

3.1.2.1 Production of the enzyme preparation

The white-rot fungus, *Trametes versicolor*, secretes cellulolytic and ligninolytic enzymes and polyphenol oxidases [Taylor *et al.*, 1987a; Taylor at al, 1987b] into its culture medium when grown in a defined liquid medium. It is believed that the defined or semi- media could constitute a model system for achieving overproduction and enhanced secretion of enzymes of biological and possibly commercial interests [Moore *et al.*, 1989]. Recently the production of ligninolytic enzymes by means of solid state fermentation has gained more attention. Although, these processes are designed mainly as methods for delignification of various materials, including sugarcane bagasse and straw [Pal *et al.*, 1995; Ortega *et al.*, 1993] and kraft pulp [Katagiri *et al.*, 1995] the secreted enzyme can be considered as a by-products of such processes, if a feasible methods for their extraction from the solid medium were found.

The enzyme reported by Khan and Overend [1990] was produced in a simple medium, reaching the maximum activity, on average 50.0 nkat/mL, after 18-23 days of incubation. A longer time of growth caused the production of an unstable enzyme preparation [Khan and Overend, 1990]. A long time of growth required to obtain the enzymes with maximum activities is characteristic for the production of peroxidases [Szklarz *et al.*, 1989; Leonowicz and Trojanowski, 1975], laccases [Rogalski *et al.*, 1990; Fåhraeus, 1952; Fåhraeus and Reinhammer, 1967], Mn-dependent peroxidases [Szklarz *et al.*, 1989; Archibald and Roy, 1992; Collins and Dobson, 1995], and ligninase [Johanson and Nyman, 1993] by the white-rot fungi when grown in the defined media. These media consist of carbon source, usually glucose, in concentrations of 0.25% [Lobarzewski, 1979], 0.5% [Lindenberg and Fåhraeus, 1952; Szklarz *et al.*, 1989; Rogalski *et al.*, 1991], and 5.0% [Fåhraeus and Reinhammer, 1967; Paszczynski and Lobarzewski, 1984], nitrogen source, usually in the form of ammonium salts, and are supplemented by different minerals, including K, Mg, Ca. Fe, Mn, Zn, and Cu.

Enzymes can also be produced in semi-defined media, such as malt extract. A three fold decrease in the time required to obtain maximum enzyme activities was reported when a 3.0% malt extract was used as a medium for the production of laccase from *T. versicolor*, as compared to the defined medium based on 2.0% glucose [Fåhraeus, 1952]. In these experiments the rates of

biomass production were apparently the same, but the obtained activities differed by 2.5 times in favour of the synthetic medium. Furthermore, in the malt-medium the enzyme activity sharply decreases after reaching the maximum, although the biomass was still produced. On the other hand, the secretion of the enzyme into the synthetic medium was accompanied by the autolysis of biomass. The appearance of enzyme in the late stages of the growth cycle is usually observed in synthetic media [Szklarz *et al.*, 1989; Fåhraeus, 1952; Rogalski *et al.*, 1990; Moore *et al.*, 1989], and could suggest that the produced enzymes are intracellular that are released from mycelium during its autolysis. However, as shown in several reports [Fåhraeus, 1952; Szklarz *et al.*, 1989], the intracellular enzymes are responsible, on average, for only a 5.0% of the total enzyme activity measured in the culture medium. This shows that the production of the enzyme does not require additional steps involving a disintegration of the produced biomass.

3.1.2.2 Enzymatic transformation of sinapic acid

To the date, the only enzymatic method to decrease the phenolic content in rapeseed/canola is the one developed in this study [Łącki and Duvnjak, 1996a; Łącki and Duvnjak, 1996b; Lorusso *et al.*, 1996]. A transformation of SA and its derivatives, such as sinapine, in the model enzymatic system by the enzyme used in the present study has been shown [Łącki and Duvnjak, 1996a; Łącki and Duvnjak, 1996c; Łącki and Duvnjak, 1996d]. It was also reported that SA can be transformed by oxidases such as inducible laccase [Bollag and Leonowicz, 1984; Rogalski *et al.*, 1991; Salas *et al.*, 1995], and mushroom tyrosinase [Choi and Sappers, 1994]. The products of these transformations were not identified, though in the latter case it was suggested that the transformation involves the formation of a quinone methide derivative of a dimer of SA. In general, the oxidoreductases catalyze oxidative coupling of two or more phenoxy radicals with the formation of oligomeric and polymeric products, mostly quinones [Lundquist and Kristerrson, 1985; Leonowicz *et al.*, 1984; Ishihara and Ishihara, 1976], from phenols and aromatic amines [Bollag, 1983; Simmons *et al.*, 1987]. Laccases from *T. versicolor* can catalyze the transformation of benzoic and cinnamic acids [Bollag *et al.*, 1982; Katase and Bollag, 1991; Leonowicz *et al.*, 1984] with the formation of quinones. Oligomeric and polymeric compounds are not the only products from these transformations. Formation of 2,6-dimethoxy-*p*-benzoquinone from 3,5-

dimethoxy-4-hydroxybenzoic acid (syringic acid) by laccase has been reported [Leonowicz *et al.*, 1984]. Since syringic and sinapic acids have the same aromatic nuclei, the formation of *p*-benzoquinone from SA by the action of oxidizing enzyme, such as PPO, can be expected. Since polyphenol oxidase also caused the formation of oligomeric products from syringic acid [Liu *et al.*, 1981], the presence of multiple products from SA conversion by enzymes from *T. versicolor* can be expected.

Chapter 4

Methods of enzyme characterization

A typical characterization of an enzyme involves determination of its kinetic parameters and their dependence on reaction conditions. These parameters are usually determined using initial reaction rates measurements, commonly referred to as enzyme activities, and/or by transient techniques that are based on the monitoring the change in the substrate concentration with time after an enzyme addition.

4.1 Initial rate measurements

The initial rate measurement methods are the most common techniques used to determine enzymatic activity [Segel, 1975]. These methods require measurements of the rate of conversion of less than 2.0% of the substrate after enzyme addition. The reaction rates obtained for different initial substrate concentrations are used to evaluate enzyme kinetic parameters and the mechanism of enzymatic transformation usually using the Steady State Approximation (SSA) approach. Even though limitations for the SSA approach are well known and recognized [Cornish-Bowden, 1979; Farrow and Edelson, 1974], the methods based on SSA are still predominant for two main reasons: the theory for SSA is simple and relatively well described, and the initial rate measurements require less specialized equipment. On the other hand, if an enzymatic reaction involves more than one substrate, the initial rate measurements do not provide enough information about the mechanism of

the reaction, unless tedious and laborious experiments with different concentration of one substrate for fixed concentrations of the other substrates are performed [Segel, 1975; Cornish-Bowden, 1979]. Furthermore, when the concentrations of substrate depend on substrate solubility then such experiments cannot be performed without the help of sophisticated equipment [Łącki and Duvnjak, 1996c], which in turn validates one of the reasons for using the initial rate measurement techniques. Considering this point and the wide accessibility of computerized equipment, the SSA approach has begun to be replaced by the transient techniques for enzyme characterization, especially due to the potentially enormous amounts of information carried by this kind of analysis [Cornish-Bowden, 1979; Frieden, 1994].

4.2 Transient techniques

The use of transient methods can be a valuable tool to evaluate enzyme reaction mechanisms, and the respective enzyme kinetic parameters [Yuo and Suelter, 1977; Halwachs, 1979; Cornish-Bowden, 1979; Orsi and Tipton, 1979; Frieden, 1994]. Application of this method is especially attractive for multi-reactant enzymatic systems. Theoretically, data regarding the progress of an enzymatic reaction obtained during only one experimental run, by monitoring changes in the concentrations of all of the substrates, should bring enough information about the reaction rates at various levels of concentration of each substrate, to evaluate the enzyme kinetic parameters. Thus, these techniques allow researchers to shorten the time of analysis and to save materials. However, transient-state data suffer severely from the so-called 'ill-conditioning' [Cornish-Bowden, 1979]. This phenomenon can be seen as the possibility of fitting the experimental results to a wide range of constants and/or equations. Consequently, wrong reaction mechanisms and wrong enzyme kinetic parameters can be obtained. In this respect, any fit to the experimental data should only be considered as one of the possible solutions of the considered problem, even though the fit may be almost perfect [Frieden, 1994].

Part II

Methodology and Experimental Aspects

Chapter 5

Methodology

This chapter includes the outline of the work performed in this study, the reasoning used to select the appropriate experiments, some theoretical considerations, and the procedure used to formulate mathematical models.

5.1 Study outline

The main objective of this research project was to decrease the phenolic content in commercial canola meal using an enzyme preparation from *Trametes versicolor*. A part of that work focused on a formulation of mechanistic or semi-mechanistic models that fit the experimental data adequately. These models were then used to mathematically describe the investigated enzymatic process for upgrading of CM. Since it would be desired to use the treated meal as a source of food-stuff, another part of this work concentrated on the elucidation of products of enzymatic transformation of sinapic acid and sinapine, in order to evaluate their impact on the meal quality.

This project was divided into three parts: enzyme production, model enzymatic system, and canola meal system. In the first part, the work performed focused on the development of a fast method for enzyme production. This was required since the enzyme preparation is not available commercially and had to be produced *in-situ*. In the second part, the enzyme produced was characterized in a "model" enzymatic system where only pure compounds were used as substrates for enzymatic reactions. In the third part, the commercial canola meal was treated with the enzyme,

and the process to decrease meal phenolic content, along with other aspects of such a treatment were investigated.

The last part also included a formulation of the mechanistic model that would described the investigated enzymatic process. This part required a knowledge of the mechanism by which sinapic acid and sinapine are enzymatically transformed. Considering the complexity of canola meal, this mechanism was elucidated in the "model" enzymatic system using sinapic acid as a substrate, and the obtained results were used to formulate a model that describes the enzymatic transformations of SA and SIN. This model was then included in the mathematical description of the enzymatic treatment of CM.

A more detailed outline of the work performed is presented in Figure 5-1.

5.2 Enzyme production

An important part of this work was to develop a fast method for the production of enzyme preparation. In the original paper, Khan and Overend [1990] reported that the production of this enzyme required a relatively long, 16-20 days, growth time. From a practical point of view such a long process was unacceptable and, therefore, a more rapid method for the enzyme production had to be proposed. The effects of initial carbon source and inoculum concentrations were evaluated as possible factors for the acceleration of the process. In addition, experiments focused on the effects of minerals concentration and the initial pH of the medium were performed. The results obtained in this part of the work should be, however, considered as a basis for a more detailed work, aiming on the optimization of the enzyme production.

5.3 Model enzymatic system

Considering that the transformations of SA and SIN by the enzyme used in the present study have not been shown yet, a separate part of this project was the characterization of this enzyme in the "model" enzymatic system using sinapic acid (SA), sinapine (SIN), sinap aldehyde (SALD), and sinapyl alcohol (SALC) as substrates.

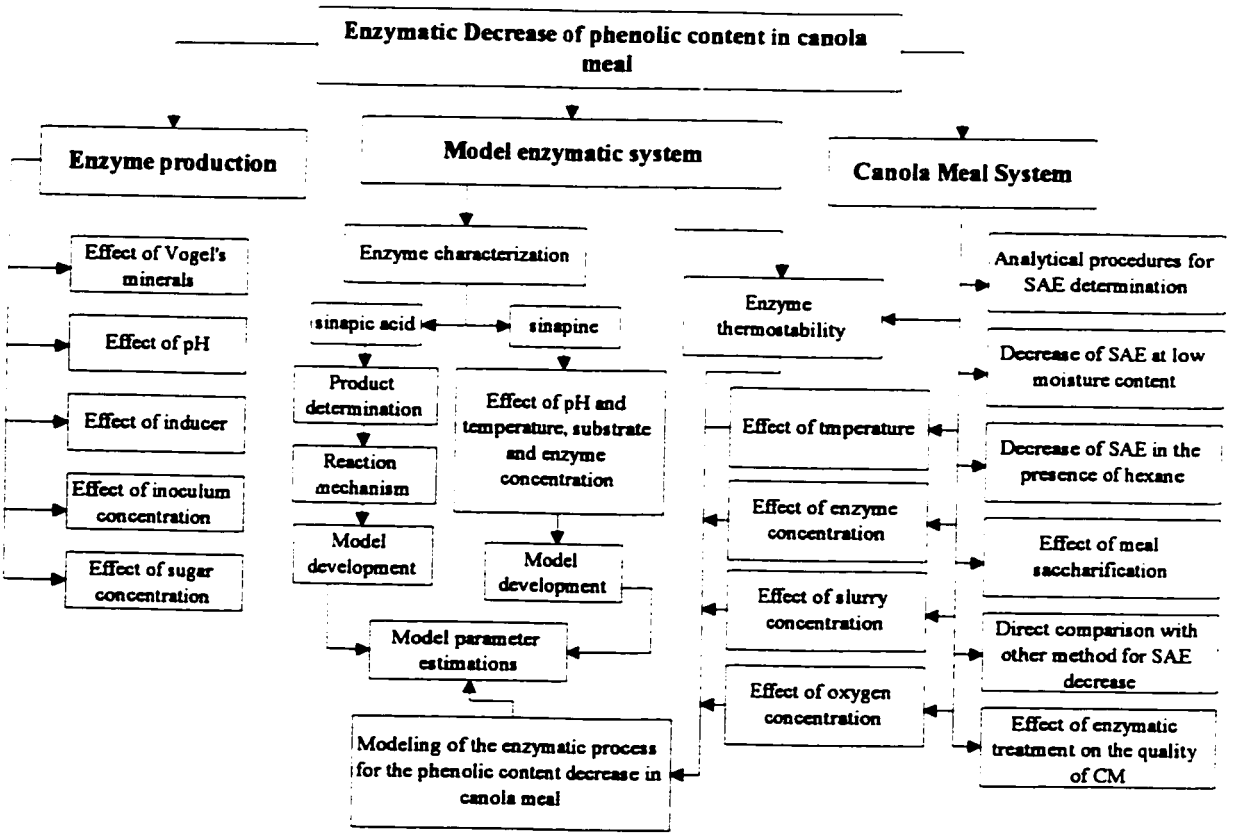


Figure 5-1. Schematic diagram representing the experimental and theoretical work done during the course of this study.

5.3.1 Combined initial rate and transient approaches applied in this work

In this work the enzyme was initially characterized using the initial rate measurement method. The measurements were performed by spectrophotometric technique in so-called "open" system. In this type of system the oxygen concentration is governed by its solubility, and since the enzymatic transformations of sinapic acid and its derivatives require oxygen as a second substrate, the results regarding the effect of temperature on enzyme activity determined using this technique could lead to wrong conclusions [Łącki and Duvnjak, 1996a]. This was the reason why the isothermal runs with different concentrations of oxygen, that are needed to elucidate the reaction mechanism, could not be performed without special experimental apparatus. To overcome such difficulties in determination of the reaction mechanism for the transformation of phenolic substrates by *Rhus* laccase, Petersen and Degn [1978] used an apparatus with a controlled composition of gas phase over the reaction mixture. An experimental constraint was that their

measurements had to be performed at relatively low oxygen concentrations to assure that the enzyme was saturated with phenolic substrate. An alternative approach is to use a system with no gas head-space which allows to attain a desired initial oxygen concentration regardless of the system temperature [Łącki and Duvnjak, 1996c]. In such a system the enzyme activity can be evaluated by varying both substrates concentrations, regardless of their magnitude. An apparatus of that kind, called here a "closed" reacting system, was designed and built during this work. It allowed simultaneous measurements of the oxygen and phenolic concentrations during the reaction at a desired constant temperature (a detailed description is provided in the next chapter). Using this apparatus, the evaluation of the reaction mechanism, and the estimation of the enzyme kinetic parameters at different temperatures were carried out using a two-step approach that combined the initial rate measurements and transient methods. In the first step, series of experiments with different initial concentrations of both substrates, phenolic and oxygen, were performed. The initial reaction rates obtained from the changes in the oxygen concentration were used to evaluate the reaction mechanism by applying the SSA approach. Based on this mechanism, a mathematical model that described the dynamics of the enzymatic transformation of SA was formulated. In the second step, the enzyme kinetic parameters in the formulated model were estimated using the transient data with respect to the changes in oxygen and SA concentrations recorded during the enzymatic transformation of SA at different reaction temperatures. This approach allowed the determination of the actual effect of temperature on the SA transformation.

5.3.2 Sinapine transformation

Considering that sinapine (SIN) is the main phenolic compound found in canola meal, a model describing its enzymatic transformation was also required. Assuming that SIN is transformed according to the same mechanism as SA, a model describing the effect of temperature, pH, both substrates and enzyme concentrations on the transformation of SIN was formulated. The estimation of the model parameters was performed using the data obtained during characterization of the enzyme with SIN as a substrate in the "open" system, taking into account the effect of temperature on the oxygen concentration. An applicability of the model to predict dynamics of SIN

conversion by the enzyme was checked by comparing model predictions with transient data collected in the "closed" system during SIN transformation.

5.3.3 Determination of the products of enzymatic transformations

The quality of the treated meal is the main issue that has to be addressed when evaluating the new method for the decrease in phenolics content in CM. In this respect it was necessary to determine what are the products of the enzymatic transformation of meal phenolics. Since canola meal is a commodity with a complex composition, the separation of products formed during the enzymatic treatment of the meal would be very difficult. Considering this fact, elucidation of the products was performed in the model enzymatic system using SA as a substrate. SA was selected because it is the major phenolic acid present in CM and is commercially available. Furthermore, the quantities of purified SIN were not sufficient to develop a method for the separation of the products from the enzymatic transformation of this compound. Moreover, the quantities of these products would not be big enough to carry out the sophisticated analytical tests, such as NMR. On the other hand, using a commercially available SA, a method to separate the products from its enzymatic transformation could be developed, and then applied to prepare enough material to perform structure elucidation studies. The elucidated products were then compared with those of sinapine transformation, detected by applying the method based on the one established with SA. In the final step of this approach, the enzymatically treated CM was analyzed for the presence of the products found in the model system. It is possible that this approach could result in non-identification of all of the products formed during the enzymatic treatment of the meal. However, considering the scope of this work it seemed appropriate to examine first the products of enzymatic transformation of the most abundant phenolics in CM in the model enzymatic system, and then to compare them with those detected in the canola meal system.

5.4 Canola meal system

For the development of the enzymatic process to decrease the phenolic content in the meal, commercially available CM was used as a raw material. Considering that laccase is almost always found in the cultures of white rot fungi, it was decided to use an unpurified enzyme preparation

from the culture of *T. versicolor* ATCC 42530. This preparation may contain at least two enzymes, laccase and the new oxygenase, required to cleavage aromatic nuclei of sinapic acid derivatives, by the mechanism explained in the previous chapters.

Since the phenolics are water soluble and can be extracted from the meal with an excess of extractant, the new process was based on the direct addition of the enzyme to the meal-buffer slurry. For this reason, the new process can be described as a combination of two separate steps: (1) the extraction of phenolics from the meal matrix, and (2) the enzymatic transformation of the extracted compounds taking place in the bulk liquid.

The simultaneous occurrence of these two steps was described by a mechanistic model that illustrates the process of extraction of a solute from a sphere into a finite volume of extracting liquid in which a chemical reaction takes place. The formulated model accounts for effects of the process variables which have the strongest influence on the process outcome, and for some physico-chemical phenomena that are occurring during the enzymatic treatment of the meal. The choice of these variables was based on the experimental results obtained from the tests focused on the determination of the extent of SAE content reduction at various process conditions. The data from these experiments were not used to estimate the model parameters, but in some cases they were used to confirm general patterns seen in the simulated results. To estimate the parameters in the formulated model, series of experiments focusing on the effects of the most important system variables were performed.

Considering that the most valuable components in CM are proteins, a part of this work dealt with the possibility of minimizing a protein loss in the treated meal, by reducing protein extraction caused by the presence of the continuous phase, *i.e.*, buffer. In these experiments, the enzymatic treatment was carried out using high concentrated meal-buffer slurries. The concentrations tested were not lower than the concentration calculated from the meal-water uptake and, therefore, in such conditions the protein content of the meal was not decreased by the extraction process.

In another set of experiments, the possibility of enhancing the new enzymatic method, by addition of an extra processing step involving meal pretreatment was considered. This step was

based on an alteration of a structure of canola meal by solubilization of cell-wall material using cellulase and xylanase.

To evaluate whether the new enzymatic treatment could be added to the already existing processes for canola meal production, the enzymatic treatment was carried out in the presence of hexane as a predominant component of the continuous phase. If such an approach proved to be successful then combination of the canola seed de-oiling process with the enzymatic treatment would produce a meal with lower phenolic content.

The final part of this work involved a direct comparison of the proposed enzymatic method with some of the reported methods that were used to decrease the phenolics content in rapeseed meals. To perform such comparisons, the commercial CM was treated using these methods, either in a single step or in combination with the enzymatic treatment. This part concluded with an attempt to evaluate the new enzymatic process based on the quality of the treated meal and the meal proteins.

In the last chapter of the thesis, the findings are summarized and a series of recommendations for the future work concerning enzymatic upgrading of canola meal is given.

5.5 Statistical Considerations

The following is the information regarding the statistical and mathematical aspects of the work, including description of minimization and numerical integration algorithms used to obtain the parameter estimates and to solve formulated models.

5.5.1 Model enzymatic system

The tests performed with the model enzymatic systems were carried out at least in triplicate. The results obtained are shown in the respective figures as the average value calculated from all replicates, with error bars representing a standard deviation.

5.5.2 Canola meal system

The tests at each process condition were carried out at least in duplicate. The results are presented as averages. The error bars represent standard deviation, however, in some cases, they are not shown for the sake of clarity of graphical presentation.

5.5.3 Mathematical Modeling

Most of the parameter estimation work performed in the course of this study was done using the software "Scientist™" version 2.0 from Micromath Scientific Software, Salt Lake City, UT. Some of the results were obtained using subroutines from the SAS Library available on the University of Ottawa network.

5.5.3.1 Minimization algorithms

Parameters in the model describing the effects of reaction conditions on the sinapine oxidase activity were estimated by a non-linear least-squares regression using the Levenberg-Marquardt algorithm. The estimation of the unknown values of the parameters in the models represented by the systems of ordinary differential equations was based on the Powell's algorithm.

5.5.3.2 Integration algorithm

To solve systems of ordinary differential equations representing different formulated models, the modified Adam's algorithm for stiff systems was used.

Chapter 6

Experimental procedures

In this chapter the considerations related to the experimental part of the work will be discussed. The experimental setups used in this study and the analytical methods used for sample analysis will be described.

6.1 Enzyme Production

6.1.1 Growth conditions

The fungus *Trametes versicolor* ATCC 42530, was grown in a semi-defined medium containing (%, w/v): glucose (2.0), yeast extract (0.5), nutrient broth (0.8) and Vogel's trace minerals (0.5). Two hundred mL of this medium, in a 500.0 mL Erlenmeyer flask, were inoculated with 10.0 mL of homogenized, freshly prepared inoculum. Prior to inoculation, the medium was sterilized at 121°C for 20 minutes. The culture was incubated in a rotary shaker at 160 rpm and at 28±2°C. The enzymatic activity was monitored using ferulic acid (FA) as the substrate [Khan and Overend, 1990]. When the activity reached the maximum, the culture was centrifuged at 5000×g, and the filter sterilized (0.45 μm) supernatant was used as the enzyme preparation. In some experiments the composition of the medium varied as described in the captions of the respective figures and tables.

6.1.2 Inoculum preparation

The fungus was maintained on agar slants containing (%, w/v): 3.0 malt extract, 0.5 yeast extract, 0.5 glucose, and 1.5 agar. Every three months the cultures were transferred to new agar slants.

Inoculum was prepared by incubating a piece of mycelium from an agar slant in the liquid medium for 6-7 days, until at least 4 fungal pellets were formed. The entire contents of the flask were then homogenized for 30 seconds, in Sunbeam blender Osterizer 8, with speed set on "liquefy". The suspension containing the homogenized pellets (biomass) was then transferred to a sterile laboratory dispenser and used as the inoculum.

6.1.3 Vogel's minerals solution

Vogel's mineral solution was prepared according to the original method [Vogel, 1959]. The trace element solution was made up as follows: in 95.0 mL distilled water, were dissolved successively with stirring at room temperature: 5.0 g of citric acid monohydrate, 5.0 g of $ZnSO_4$ $7H_2O$, 1.0 g $Fe(NH_4)_2(SO_4)_2$ $6H_2O$, 0.25 g of $CuSO_4$ $5H_2O$, 0.05 g of $MnSO_4$ $1H_2O$, 0.05 g of H_3BO_3 anhydrous, and 0.05 g of Na_2MoO_4 $2H_2O$. The resulting volume was adjusted to 100.0 mL and 1.0 mL of chloroform was added as a preservative. The solution was stored at room temperature.

6.1.4 Inducer (xylidine) solution

The solution of xylidine was prepared according to Rogalski *et al.* [1990]. One hundred twenty one point two mg of 2,5-xylidine was dissolved in 5.0 mL of 50.0% ethanol. This solution was added as the inducer to stimulate laccase production. The concentration xylidine in the culture medium was 0.2 mM.

6.2 Model enzymatic system

6.2.1 Enzyme characterization a steady state approach

Enzyme activity was measured spectrophotometrically by following the loss in absorption of SA and SALD at 310 and 340 nm, respectively, using a Beckman DU 640 spectrophotometer equipped with temperature controller. The basic reaction system consisted of 2.5 mL 0.05-M citrate-phosphate buffer [McIlvaine, 1921] of a chosen pH, 0.5 mL substrate solution, and 10 μL of the enzyme or its dilution. The reaction conditions are specified in the legend of the respective figures and tables.

The sinapine oxidase activity was also measured spectrophotometrically, but at 328 nm. The reacting system consisted of: 0.7 mL (0.5-mM) of SIN solution, 2.2 mL of phosphate-citrate buffer (pH range tested from 2.5 to 7.3), and 0.1 mL of enzyme preparation. The reaction was

carried out in 20.0 mL test tubes placed in a water bath at 283.2 K to 353.2 K. The buffer and sinapine solutions were heated up to the reaction temperature prior to the addition of enzyme. The reaction was stopped by adding 0.1 mL of 66.0% trichloro acetic acid (TCA). The initial absorbance of the reacting solution was measured using the same system, but TCA was added before the enzyme. The enzyme activity was expressed in terms of nanokatals (nmol/s). The effect of pH was examined at each temperature level. Changes of pH due to the temperature of the system were taken into account using the automatic temperature compensation, ATC, mode on the pH meter.

The enzyme activities with each substrate were evaluated from the linear part of the progress curves using Eq.(6.1)

$$v = 10^6 \frac{(A_{in} - A_t)}{\Delta t} \frac{1}{\varepsilon} \frac{V_{sys}}{v_e} Dil \qquad (6.1)$$

where v is the enzyme activity (nkat mL^{-1}), A_{in} and A_t are the initial and final absorbancies, respectively, Δt is the reaction time (s), V_{sys} is the volume of the system (mL), v_e is the volume of the enzyme added (mL), *Dil* is the enzyme dilution factor, ε is the molar absorptivity (SA, 19690 $M^{-1} cm^{-1}$; SIN, 22169 $M^{-1}cm^{-1}$; SALD, 23459 $M^{-1}cm^{-1}$; FA, 13650 $M^{-1}cm^{-1}$).

6.2.2 Enzyme thermal stability

The crude enzyme preparation was diluted to 5.0% with either distilled water or citrate-phosphate buffer of a chosen pH. The solution was exposed to a certain temperature in a temperature controlled water bath. After a predetermined time, samples were withdrawn and the activity was measured using sinapine as a substrate. The results are expressed as a percentage of the initial activity measured just before the beginning of incubation.

6.2.3 Enzyme characterization a transient approach

Dynamics of the enzymatic transformations of SA and SIN were investigated using a transient technique. The experimental apparatus used for these enzymatic assays, which in this study is called a "closed" system, is presented in Figure 6-1. A 125-mL, custom-built, glass reactor with a magnetic stirrer and a water jacket, to maintain a constant mixing and a constant temperature during the reaction, respectively, was filled with a 0.45-mM solution of sinapic acid in 0.05-M pH-3.5 phosphate-citrate buffer or 0.5 mM solution of sinapine bisulfate in 0.05-M pH 4.5

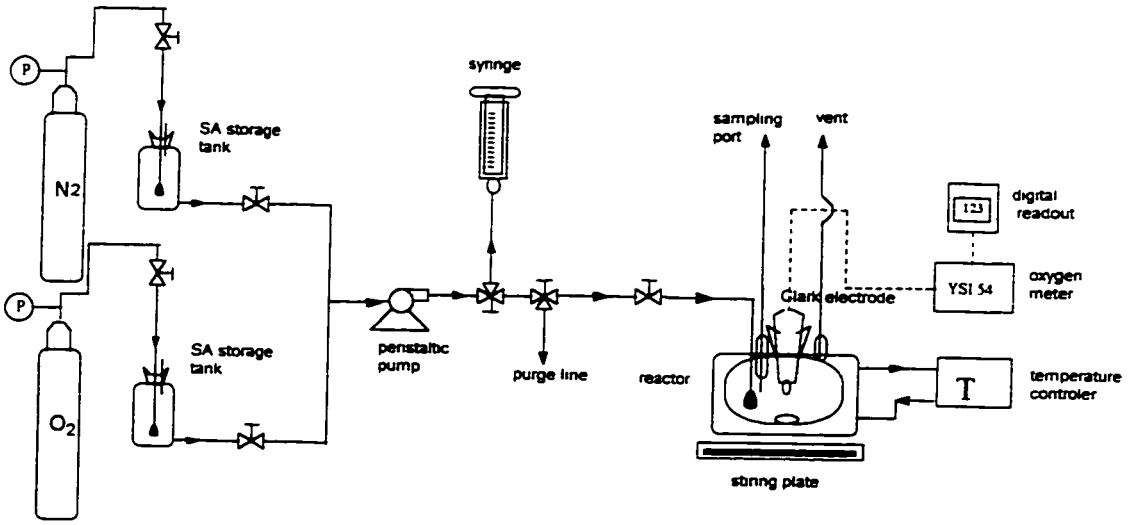


Figure 6-1. Scheme of the experimental setup: "closed" system.

phosphate-citrate buffer. A desired initial oxygen concentration was obtained by pumping respective amounts of oxygen- and/or nitrogen-sparged substrate solutions into a gas-tight syringe and transferring the mixture into the air-free, zero-head-space reactor. A final adjustment of the initial oxygen concentration was obtained by pumping either nitrogen- or oxygen-containing substrate solutions through the reactor. Using this method, the initial oxygen concentrations can be obtained with an accuracy of 0.1 ppm. Oxygen leaks to or from the system were prevented by using water-sealed inlet, outlet, and sampling ports that were designed in this work. The reactor temperature was controlled by a controller (Haake F3) that circulated water through the jacket. The reaction was started by adding 0.1 mL of enzyme stock solution through the sampling port. Changes in the oxygen concentration were measured using a Clark electrode connected to a YSI 54 dissolved-oxygen meter with a digital readout or to a PC data acquisition system. Changes in SA or SIN concentration were measured as follows: 20-μL samples of the reacting solution were withdrawn from the reactor through the sampling port and transferred into 50-μL sampling vials, with septum caps, containing 5-μL 1-N HCl to stop the reaction. The samples were analyzed for sinapic acid using high performance liquid chromatography (HPLC) Method A (for a detailed description see section 6.4.9.1), and for SIN using HPLC method B. HCl did not affect the stability either of sinapic acid or sinapine in the samples. During a run, sampling caused the total volume of the reacting mixture to change less than 0.5%.

As a control, to determine the extent of oxygen disappearance due to the Clark's electrode, its consumption was measured in a reactor filled with SA solution in the absence of enzyme at different temperatures for at least 30 hours.

In the case of the so called "open" system, the reaction was performed in a glass beaker equipped with a water jacket. The oxygen mass transfer coefficient, k_La, which describes the oxygen transfer from the air to the liquid, was evaluated by monitoring changes in the oxygen concentration in the bulk enzyme-free SA solution which was previously sparged with nitrogen.

6.2.4 Product determination

6.2.4.1 Enzymatic transformation of sinapic acid

Two hundred mL of 0.45-mM aqueous SA solution (resulting pH 3.5) with the undiluted enzyme preparation containing 16 nkat of the polyphenol oxidase were incubated (30°C, 100 rpm) in a 500 mL Erlenmeyer flask, closed with a foam stopper. After a predetermined time, the reaction mixture was extracted (30 min) with 400 mL of methylene chloride (MeCl). The extracted organic phase was concentrated (37°C) under vacuum to a volume of 3 mL using a rotary evaporator. The concentrates were stored at room temperature in closed tubes covered with an aluminum film.

6.2.4.2 Enzymatic transformation of dehydrodisinapic acid dilactone

A stock solution of r-1H2c,6c-bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione, DAD, was prepared daily by dissolving 3.5 mg of this compound (separated in this work) in 20 mL of distilled water. The resulting solution was stored at 4°C between tests. Enzymatic transformation of DAD was examined in a spectrophotometric cuvette by following the increase in its absorbance (519 nm) using a Beckman DU 640 spectrophotometer equipped with a temperature controller, for up to 10 minutes after the addition of the enzyme. The reaction system consisted of 2.5 mL citrate-phosphate buffer (pH ranging from 2.7 to 6.2), 0.2 mL substrate solution prepared from its stock solution, and 10 μL of the enzyme preparation (concentration of 40 nkat/mL or appropriate dilution). The enzyme activities were evaluated from the linear part of the progress curves. Thermolysis of DAD was carried out at pH 4.5 in a spectrophotometric cuvette placed in a temperature controlled cuvette holder. The extent of thermolysis of DAD was evaluated by monitoring the change in the absorbance at 337 nm.

6.2.4.3 Fractions collection

The products of SA transformation obtained after 3 hour-long enzymatic reaction were separated using HPLC Method A (for detailed description see section 6.4.9.1), and the fractions responsible for particular peaks were collected into methylene chloride from the column effluent. The organic phase from each fraction was withdrawn with Pasteur pipettes and concentrated under vacuum. The purity of each fraction was confirmed using HPLC.

6.2.4.4 Determination of the product yields

To determine the yields for the products formed during enzymatic transformation of SA, a 350 mL of 0.5 mM SA was transformed at 27°C using 0.5 mL of enzyme stock solution. After a predesignated time, the reacting solution was extracted with MeCl (3 times 300 mL) and the combined organic phases were evaporated under vacuum to dryness. The mass of the dried sample was used to calculate the yield of the products extractable with the organic solvent. The water phase remaining after MeCl extraction was centrifuged and a precipitate was collected. The supernatant was concentrated in a rotary-evaporator under vacuum at 40°C and lyophilized using Uni-Trap Model 10-100. The mass of the dried samples was used to calculate the yield of the products that were not extracted with the organic solvent.

6.2.4.5 MS, FTIR, NMR and X-Ray analysis

MS (Mass spectrometry)

The mass spectra of the separated products were obtained by electron impact mass spectronic analysis (70eV) with sample introduction by direct insertion probe using VG Analytical Model 7070E mass spectrometer at a source temperature of 250°C.

NMR (Nuclear Resonanse Magnetic spectrometry)

Proton ^{1}H and carbon ^{13}C NMR spectra of the analyzed compounds dissolved in CD_2Cl_2 were obtained using a Bruker AMX-500 NMR spectrometer.

FTIR (Fourier Transformed Infra-Red spectrophotometry)

Infrared spectra of the collected fractions were taken using a Perkin-Elmer 1500 instrument with a resolution of 4 cm^{-1}. The samples were transferred to solid NaCl windows using MeCl.

X-Ray (X-Ray Diffraction Crystallography)

The details of X-Ray analysis are given in Appendix E.

6.3 Canola Meal System

6.3.1 Decrease of phenolic content in canola meal - basic approach

The enzymatic process to decrease the phenolic content in a commercial CM was carried out in two basic systems.

System I: 1 g of CM was placed in a 50 mL Erlenmeyer flask, the citrate-phosphate buffer of a chosen pH was added and the mixture was supplemented with an enzyme solution of fixed activity (for each gram of meal one milliliter of the enzyme preparation was added). A desired enzyme concentration was obtained by dilution of the enzyme preparation with distilled de-ionized water. A constant temperature was obtained by using a temperature controlled water bath, Fisher A22. The reaction was stopped by the addition of 66.0% TCA using a volume equal to that of the enzyme addition. The suspension was then centrifuged for 20 minutes at 5000g and 5°C. After decanting the supernatant, the residue was oven dried (100°C) for 3 hours. A sample of the dried meal was analyzed for SAE content using one of the analytical methods (section 6.4.5), and the extent of phenolic concentration decrease was calculated using the initial meal SAE content.

System II: 0.5 g of CM was placed in 14 mL centrifugal tubes, the citrate-phosphate buffer of a chosen pH was added and the mixture was supplemented with 0.5 mL of an enzyme solution of fixed activity. The process was carried out at a constant temperature using a custom made temperature controlled air chamber with a rotary tube rack mixer, Fisher Roto-rack model 440. The reaction was stopped adding 0.5 mL of 66.0% TCA. In some tests the reaction was stopped by rapid cooling of the reacting solution in an ice-bath. The suspension was centrifuged for 20 minutes at 5000×g and 5°C. After decanting the supernatant, the residue was extracted with MeOH and the SAE content was measured using one of the analytical methods. The supernatant was analyzed for protein and carbohydrates content.

6.3.1.1 Effect of pH

The effect of pH of the buffer on the SAE content decrease was tested in system I by varying the pH between 3.0 and 7.2. The process was carried out at 50°C for 1 hour, using 20.0% (w/v) meal-buffer slurry concentration and 20.0 nkat of enzyme per gram of the treated meal. A deactivated enzyme was used to determine the effect of the buffer pH on SAE extraction.

6.3.1.2 Effect of temperature

The effect of temperature on the rate of SAE content decrease was tested in system I by measuring the concentrations of the residuals SAE in the meal after a 1 hour-long treatment of 20.0% meal-buffer slurry using two enzyme concentrations of 20.0 nkat/g and 1.0 nkat/g. pH of the buffer was 4.5. Temperatures tested were between 10°C and 70°C. At each tested temperature, a control run was performed using the deactivated enzyme.

The effect of temperature on the dynamics of SAE decrease was studied using system II. The meal and enzyme concentrations were 10.0% (w/v) and 4.0 nkat/g, respectively. pH of the buffer was 4.5. The tests were carried out at 20.0°C, 35.0°C, 50.0°C and 75.0°C.

6.3.1.3 Effect of enzyme concentration

To investigate the effect of enzyme concentration on the rate of SAE content decrease, the enzymatic process was carried out in system I. The rate was determined from the changes in SAE content after 1 hour long treatment of 20.0% (w/v) meal suspension at 35.0°C. The enzyme concentrations tested varied from 0.0 to 200.0 nkat per gram of the treated meal.

The dynamics of SAE decrease for different enzyme concentrations was investigated using system II at 50.0°C. Meal concentration was 10.0% (w/v) and pH of buffer was 4.5. Enzyme concentration tested included 0.0, 2.0, 4.0, 20.0, 40.0 nkat per gram of treated meal.

6.3.1.4 Effect of slurry concentration

The effect of meal-buffer slurry concentrations on the SAE decrease was examined in system I. Two series of experiments with meal concentration ranging from 1.0% to 33.0% were performed. In the first one, the enzyme concentration in the liquid phase was kept constant at 4.0 nkat/mL, while in the second series the enzyme concentration was kept proportional to the meal concentration, *i.e.*, kept constant at 4.0 nkat per gram of the treated meal. As controls, system with deactivated enzyme were analyzed. In all cases rates of SAE decrease were determined from the changes in SAE concentration after 30 min treatment at 50.0°C. pH of buffer was 4.5.

6.3.1.5 Effect of oxygen concentration

To examine the effect of oxygen concentration on the decrease in SAE content in CM, the dynamics of this process was followed for different initial concentrations of oxygen using system II with a following modification. The 10.0% (w/v) slurry of CM was placed in 14 mL centrifuge tubes, with screw caps equipped with septum. Using a syringe with a needle, the composition of the

head space in each tube was changed by first withdrawing a given volume of it and then replacing it with the same volume of oxygen or nitrogen. Such systems were supplemented with enzyme, 2.0 nkat per gram of treated meal, and the process was carried out at 50.0°C. pH of the buffer used was 4.5.

To determine an overall stoichiometry for the enzymatic transformation of SAE, batch experiments with different amounts of oxygen available in the system were performed. Different amounts of oxygen in the system were obtained by varying the volume of the head gas space in the reactor (culture tube with septum cap) changing the volume of the 10.0% (w/v) of canola meal-buffer-enzyme suspension.

6.3.1.6 Effect of particle size and intensity of agitation

To examine the effects of external and internal diffusion on the SAE decrease in CM, the enzymatic process was carried out using system II. The effect of external diffusion was tested by changing the intensity of the agitation during the treatment of 10.0% (w/v) meal suspension at 50.0°C using 4.0 nkat of enzyme per gram of the meal. pH of buffer was 4.5.

The effect of internal diffusion was tested by following the dynamics of SAE decrease in different fractions of the meal obtained from sieve analysis. The process was carried out using system II at 50.0°C and 10.0% (w/v) meal concentration with 4.0 nkat of enzyme per gram of the meal.

6.3.2 Effect of meal solubilization

To examine the possibility of the proposed process enhancement by altering the structure of the meals' cell-wall, the CM was treated with cell-wall solubilizing enzymes prior to or during the treatment with phenol oxidase. Cellulase enzyme preparation and Iogen GS35, a stabilized liquid xylanase preparation formulated and standardized for use in pulp bleaching, were obtained from Iogen Corporation, Ottawa, On., Canada. The cellulase had an activity of 166.0 Filter paper units (FPU) per mL, whereas the GS35 had the xylanase activity of 3600 units and cellulase activity of 20.0 FPU. The cellulase and GS35 were stabilized by 0.65% and 0.6% of sodium benzoate, respectively. Both preparations were in 30.0% sucrose solutions.

Saccharification of canola meal was carried out in culture tubes with screw caps. Each tube contained 1 g of canola meal, 8.0 mL of 0.05-M citrate-phosphate buffer (pH 4.5), 1 mL of oxidase with activity of 40 nkat/mL, and 1 mL of an appropriate dilution of either cellulase or GS35 (xylanase) enzyme preparations. The tubes were incubated at 50°C with continuous mixing

for a designated period of time using the Roto-rack and a thermostatic air chamber. After incubation, the tubes were centrifuged at 3000×g and 5°C for 20 minutes and the free and total sugars were estimated in the supernatant before and after hydrolysis, respectively. Hydrolysis was carried out in 0.4-N HCl at 100°C for 15 minutes and the hydrolyzed mixture was neutralized with NaOH after a rapid cooling in an ice-bath. The sugars were estimated using dinitrosalicylic acid (DNS) method. In some tests the tubes with CM were autoclaved at 121°C for 15 minutes prior to the addition of the enzymes. Each of the tests was carried out in triplicates.

The process to decrease the SAE content of the meal was carried out in culture tubes at 50°C for different lengths of time. A 0.5 mL of a proper dilution of PPO and a 0.5 mL of a proper dilution of one of the wall solubilizing enzyme preparations were added to each tube containing 11.0% (w/v) meal suspension. The meal residue obtained after a centrifugation of the treated suspension was analyzed for SAE content using the chemical spectrophotometric method [Mueller *et al.*, 1978a]. The results were adjusted for the interference from reaction products according to Łącki and Duvnjak [1996b].

6.3.3 Enzyme thermal stability in the presence of canola meal

To evaluate the effect of canola meal and canola proteins on the thermal stability of the enzyme, 1 mL of the enzyme preparation was added to 14.0 mL centrifuge tubes containing either 9 mL of a 11.1% CM suspension in a citrate-phosphate buffer of a chosen pH or 9 mL of the solution of the canola meal protein. The canola meal proteins were previously extracted from the meal under the same conditions as those used in the enzyme incubation tests. In some tests, the canola meal protein were replaced with bovine albumin. The incubation was carried out at constant temperature in a custom made temperature controlled air chamber with a rotary tube rack mixer, Fisher Roto-rack model 440. After a predetermined time, 10 μL of the incubated solution were withdrawn from the tubes and the activities were measured spectrophotometrically using SA as a substrate. The results are expressed as a percentage of the initial enzyme activity determined before the beginning of the incubation.

6.3.4 Decrease in phenolic content at low moisture level

The enzymatic process to decrease the SAE content in canola meal at low moisture content was carried out at different temperatures inside a single wall gravity-convection laboratory oven. Temperatures tested were 50°C, 70°C, 90°C and 110°C. Aluminum dishes were used as open batch reactors. Each dish contained 1.0 or 2.0 gram of canola meal and an enzyme-buffer solution

with a given enzyme concentration. The pH of the citrate-phosphate buffer was 4.5. The meal-enzyme-buffer slurry was occasionally mixed using a glass rod. After a designated time, a dish was removed from the oven and a sample for SAE content determination was taken. The moisture content of the meal was measured from the change in the mass of each dish before and after the sampling. In some experiments an additional amount of water was added to the meal during the process. The addition was performed in single doses after designated periods of time. The new moisture contents of the meal were calculated from the water mass balance. To determine the SAE content in the treated meal, a sample of the meal taken from a dish was extracted with 20.0 mL of 1.0% HCl in MeOH and the SAE content was measured using the chemical spectrophotometric method. The mass of the analyzed sample was determined from the change in the mass of the aluminum dish before and after the sampling. The SAE content was expressed on dry basis.

The enzymatic process at 15°C and 25°C was carried out using a batch reactor made from a laboratory filter-funnel and a plexi-glass column (internal diameter: 3.7 cm, height: 9.5 cm) equipped with a water jacket connected to a Haake F3 temperature controller to maintain a constant temperature. The process was started by adding the enzyme-buffer solution to the reactor filled with CM and mixing the slurry with a glass rod. The sampling procedure consisted of mixing of the meal and withdrawing two samples. One sample was used to determine the SAE content and the other to measure meal moisture content. Between sampling the meal was left undisturbed. In the experiment with an increased oxygen concentration, the stream of air (25.0 mL/min) was introduced through the bottom of the reactor and the experiment was carried out as described above. In order to use a dry air at process temperature, before entering the column, the air was passed through a laboratory heat-exchanger filled with an anhydrous $CaSO_4$.

In the experiments with a constant moisture content, the aluminum dishes were placed inside an autoclave with the operating temperature set at 100°C. The door of the autoclave was left open and a tap-water was added continuously to compensate for the water evaporation. The SAE content was determined as in other experiments.

6.3.5 Decrease of phenolic content in the presence of hexane

To investigate a possibility to incorporate the enzymatic step to already existing industrial processes for a meal production, the enzymatic process was carried out in the presence of hexane. The basic investigated system consisted of: 1.0 g canola meal, 10.0 mL of hexane and 1.0 to 3.0 mL of enzyme preparation with a desired activity. The process was carried out in 50.0 mL Erlenmeyer flasks. After the addition of enzyme to the preheated meal-hexane mixture, the flasks

were closed with rubber stoppers and the incubation was carried out at a constant temperature with the continuous mixing using Fisher Scientific Blue M water-bath shaker. After a designated time, the reaction was stopped with 1.0 mL of 66.0% TCA and the mixture was centrifuged at 1500×g for 10 min. The meal residue was dried for 3 hours at 100°C and the SAE content was determined using the chemical spectrophotometric method.

6.3.6 Decrease of phenolic content using reported method

To evaluate the new enzymatic treatment a direct comparison of its efficiency with the results obtained using other reported methods for phenolic content decrease was performed. A short description of each of the tested method is provided later in the text, when the respective results are presented.

6.4 Analysis of samples

6.4.1 Biomass

The content of each flask was filtered through the filter paper Whatman 2, and the collected biomass was washed with an excess of distilled water. The filter paper with the biomass was dried in a laboratory oven at 90°C overnight. The sample was then weighed and the result adjusted for the mass of filter paper.

6.4.2 Carbohydrates

To determine a total carbohydrates content in canola meal the following procedure was used: 3.0 g of the meal were boiled with 50.0 mL of water and 10.0 mL of 12-N HCl. The extraction was carried out in a 100.0 mL boiling flask equipped with a condenser, for 2 hours. After cooling, the sample was centrifuged at 3000×g at 5°C, and the supernatant was analyzed for sugar using DNS method.

The concentrations of free water-soluble sugars were determined in the continuous phase using DNS method. Total water-soluble sugars were measured in the same sample, after its 10 minutes long acid hydrolysis in boiling 1-N HCl [Gattinger *et al*, 1990].

6.4.3 Meal water uptake

To determine a meal water (buffer) uptake, 1.0 g of the meal was mixed with different volumes (1 to 100 mL) of the citrate-phosphate buffer of pH 4.5. After 1 hour of incubation with occasional mixing, the suspension was separated by centrifugation at 5000×g for 10 min, and the

supernatant was collected. The meal water uptake, MU, was calculated as a difference between the initial volume of buffer added and the volume of collected supernatant.

6.4.4 Moisture

The moisture content of canola meal was determined as a change in the mass of the sample before and after drying at 90°C for 12 hours.

6.4.5 Phenolics

The following is a description of the methods used to measure the concentration of the phenolic content in the meal. The basic procedure that was followed during phenolics measurements is shown in Figure 6-2.

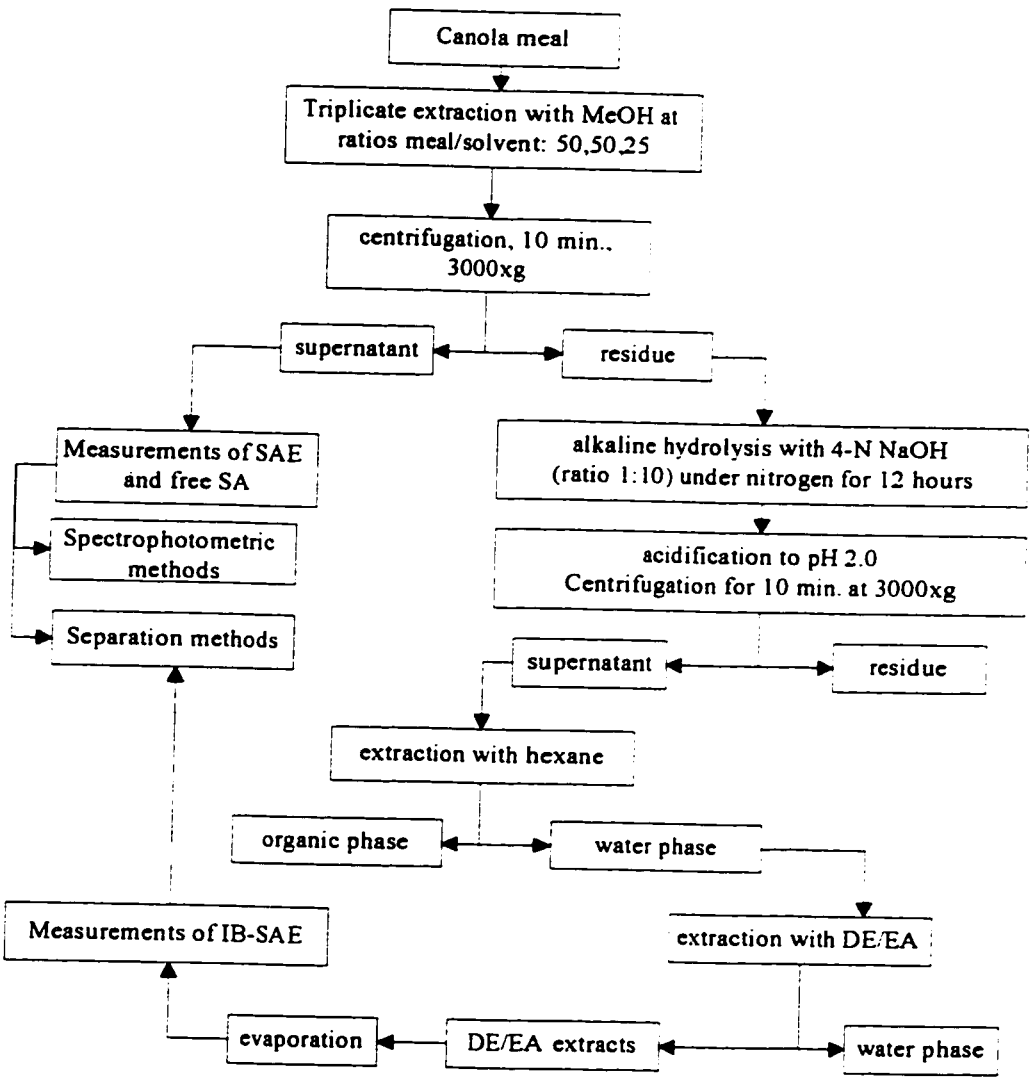


Figure 6-2. Procedure for the extraction and separation of free, esterified, and insoluble-bound phenolic compounds in the treated and untreated canola meal.

6.4.5.1 Extraction of phenolics from canola meal

Soluble phenolics: Free phenolic acids and soluble SAE were extracted from CM with boiling methanol. Meal was placed in 100.0 mL boiling flasks, and 2.0% v/v HCl in methanol was added. The flasks were equipped with condensers and placed in a 90°C water bath for 30 minutes. The extraction was repeated three times with the MeOH- meal ratios of 50, 50, and 25. After each extraction, the suspension was centrifuged for 10 min at 3000×g. The combined supernatants were analyzed for free sinapic acid, sinapic acid esters and sinapine content using either direct or chemical spectrophotometric method, and/or HPLC Method B (for a detailed description see section 6.4.9.2).

Insoluble-bound phenolics: The residue (meal) after methanol extraction was hydrolyzed with 4N NaOH under nitrogen for 12 hours at room temperature using the ratio of meal to base of 1/20. The hydrolysate was acidified to pH 2.0 using 6.0 mL of 8.0-N HCl and centrifuged at 3000×g for 10 min. The obtained supernatant was extracted 3 times with an equal volume of hexane to remove free fatty acids and other lipid contaminants. The water phase was then extracted twice with an equal volume of diethylether-ethyl acetate, DE/EA. The concentration of free sinapic acid originating from insoluble-bound esters was measured using HPLC.

6.4.5.2 Sinapine

Concentration of sinapine (SIN) in methanol extract was determined by the Reinecke salt method [Austin and Wollf, 1968]. One mL of the methanol extract was evaporated to dryness in a rotary evaporator at 50°C. The residue was dissolved in 3.0 mL of distilled water and chilled for 5 min. Two mL of a saturated solution of Reinecke salt (0.5 g/100 mL) was then added and the solution was placed in an ice bath for 90 min. The precipitate was separated by centrifugation at 3000×g and 5°C for 20 min. After decanting, the residue was redissolved in 10.0 mL of 2.0% HCl in methanol. Two mL of this solution were transferred to a glass cuvette and the absorbance was recorded at 400 nm. One mL of 1N NaOH was then added and final absorbance was recorded within 5 second of the base addition. If the readings were too high the supernatant was further diluted with acidified methanol. The blank was prepared using methanol treated in the same way. Sinapine content was calculated using a standard curve prepared with sinapine bisulfate. The relation between absorbance and purified sinapine bisulfate was linear in the range of 0-6 μg per mL of methanol solution (Appendix C).

6.4.5.3 Sinapic acid esters

All sinapic acid esters (SAE) were determined by Mueller's method [Mueller *et al.*, 1978a]. Fifty μl of the methanol extract from CM were diluted with 2.0 mL of acidified methanol and the amount of sinapic acid esters was measured and calculated using the procedure for sinapine determination, with the following modification [Mueller *et al.*, 1978a]: the concentration of SAE was evaluated based on ΔA instead of from the final absorbance (Eq.(6.2)):

$$\Delta A = A_{final} - \frac{2}{3} A_{initial} \tag{6.2}$$

where: $A_{initial}$ and A_{final} are the absorbance of the sample before and after base addition, respectively.

The numerical factor in Eq.(6.2) accounts for sample dilution after the addition of NaOH.

6.4.6 Proteins

The protein concentration in the sample was measured using the modified Lowry method [Schacterle and Pollack, 1973]. One mL of the sample was mixed with 1 mL of the copper reagent and allowed to stand undisturbed for 10 min. Four mL of the phenol reagent were then added and the mixture was incubated at 55°C for 5 min. The absorbance of rapidly cooled sample was read at 650 nm, against water treated the same way as a sample. To prepare the alkaline copper reagent 5.0 g of sodium hydroxide, 0.25 g potassium tartrate and 0.125 g copper sulfate were dissolved in 250.0 mL of a 10.0% sodium carbonate solution To prepare the phenol reagent, commercially obtained the Folin-Ciocalteu phenol reagent was diluted 18 times. The phenol reagent had to be prepared daily, whereas the alkaline copper reagent was stable for at least 30 days at room temperature.

To determine the protein content of canola meal, 3.0 g samples of the meal were boiled with 50.0 mL 1-N NaOH in a boiling flask equipped with a condenser. After cooling and centrifuging, the appropriately diluted supernatant was used for the determination of the meal protein content.

6.4.7 Functional properties of protein isolates

The nitrogen solubility was determined according to AACC [1971] at the Ministry of Agriculture Laboratories, Ottawa, On.

The whipping capacity was expressed as percent volume increase of 50 mL of 3.0% sample dispersion whipped with a high speed Sunbeam electric mixer for 6 minutes, and the foam stability as a volume of foam remaining after a standing period of up to 120 minutes [Thompson *et al.*, 1982].

The colour of the isolate was described by matching it with a sample of the colour displayed on the SVGA monitor using the 800×600 pixel display. The colour was expressed by the values of Red (R), Green (G) and Blue (B) intensities for a 256 colour palette. The principle of this procedure is described below using, as an example, the steps required to see the given colour using Microsoft PowerPoint v.7: (1) check if the computer display is configured at a 256 colour palette and 800×600 desktop area; (2) start PowerPoint, open a blank presentation, insert your favorite shape *i.e.*, circle, star, box etc.; (3) from the Toolbar choose in order: Format, Colors and Lines, Fill, Other color..., and finally a Custom panel; (4) using a mouse choose from the colour panel the closest colour that match the colour of the protein isolates; (5) read of the values from respective cells for the Red, Green, and Blue intensities; (6) to see the reported colour enter the reported values for R, G, B intensities into respective cells and observed a small window called New color.

6.4.8 Reducing sugars

The concentration of reducing sugar was determined using the dinitrosalycilic acid (DNS) method [Miller, 1959; Weiner, 1978]. One mL of the sample, containing no more than 0.5% (w/v) of mono-saccharide, was mixed with 3.0 mL of the DNS reagent and boiled for 5 min in a water bath. After immediate cooling, 20.0 mL of distilled water was added to the sample and the absorbance was read at 600 nm against water treated the same way as the sample.

6.4.9 High-performance liquid chromatography methods

High-performance liquid chromatography (HPLC) separations were performed on a Waters Associates HPLC system equipped with a 600E system controller, a U6k injector and a Waters 486 tunable absorbance detector. The ultraviolet-visible spectra of the detected compounds were obtained with a Waters 490 photo diode array (PDA) detector which in some runs replaced a Waters 486 detector. The acquired data were analyzed using the Millennium Waters chromatography system manager, Millennium v. 2.00.

6.4.9.1 Method A: Sinapic acid

Fifty μL of concentrated extracts were separated on a Spherisorb ODS2, 5 μm, 250 × 4 mm, cartridge column purchased from Hewlett-Packard. The mobile phase consisted of 0.005% phosphoric acid (A) and acetonitrile (B). The mobile phase flow rate was 1.0 mL/min. The following mobile phase gradient method was used: linear from 100.0% A to 70.0% A and 30.0% B in the first 17 minutes, kept at this composition for the next 5 minutes, then brought to 100.0% B at 30 minutes using curve #8 from the Waters gradient curve tables. The total run time was 51 minutes. The column was equilibrated at initial conditions for 15 minutes before a new injection was performed. The samples were detected at 280 nm wavelength. The separation was performed at 30°C.

6.4.9.2 Method B: Sinapine and SAE

The methanol extracts from the meal were analyzed for sinapine, sinapic acid and other SAE using the separation method of Clausen *et al.* [1983]. This method was slightly modified to accommodate for the different HPLC equipment. A 120 × 4.6 mm I.D., Supelcosil LC-8 (5μ) column was used. The mobile phase consisted of the solvent A (0.01 n-dibutylamine and 0.01 M sodium heptanesulfonic acid (pH 2.2)) and solvent B (0.005 M n-dibutylamine and 0.005 M sodium heptanesulfonic acid (pH 2.2) modified with 50.0% acetonitrile). The composition of the mobile phase was changed linearly for 30 min from 2:8 to 7:3 of solvent B to solvent A, then was kept at the initial composition for 10 min. The flow rate was set at 1.0 mL/min. The data were acquired at a 328 nm wavelength. The temperature of separation was 50°C. The size of the sample was 10 μL.

6.5 Chemicals

The alphabetical list of chemicals used in this study together with their sources are given in Table 6-1.

Sinapine was extracted from commercial canola meal and purified as bisulfate according to Clandinin [1961]. The yield was 0.297 g from 470.0 g of the canola meal. The melting point of purified crystals was 187-187.5°C, which is the same as the literature values [Clandinin, 1961; Schwarze, 1949]. A sample of this compound (10 mg) was also provided by professor Thomas Vogt from the Department of Plant Science at the University of British Columbia.

Table 6-1. List of chemicals used during the course of this research.[a]

A	Albumin bovine (Sigma), Acetonitrile (OmniSolv-BDH)
B	Boric acid (Fisher)
C	Citric acid anhydrous (Sigma), Catechin (Sigma), Caffeic acid (Sigma), Calcium Chloride (Sigma), p-coumaric (Sigma), Chloroform (Sigma)
D	Dichloromethane (OmniSolv-BDH), 3,5-dinitrosalicylic acid (BDH), DI-n-butylamine (Sigma)
E	Ethanol (BDH)
F	Ferrous ammonium sulfate (Sigma), Ferulic acid (Sigma)
G	D-Glucose (BDH)
H	1-Heptanesulfonic acid (Sigma)
M	Methanol spectrophotometric grade (Sigma), Methanol (BDH), Magnesium sulfate heptahydrate (Sigma), Manganium sulfate (Sigma), Methylene Chloride (BDH)
N	Nutrient Broth (Mikrobiologie-BDH)
P	Potasium sodium tartrate (BDH, Sigma), Protocatechuic acid (Sigma), Potasium thiocyanate (Sigma), Potasium phosphate monobasic (Sigma), Phenol reagent (BDH), Phosphoric acid - syrup (Sigma)
R	Reinecke salt (Sigma)
S	Sucrose (AnalaR-BDH), Syringic acid (Sigma), Syringaldazine (Aldrich), syringaldehyde (Aldrich), Sinapic acid (Aldrich), Sinap alcohol (Aldrich), Sinap aldehyde (Aldrich), Sinapine (this work), Sodium hydroxide (BDH)
T	Trichloroethylene (Fisher), Trichloroacetic acid (AnalaR BDH), Tannic acid (Sigma)
V	Vanilic acid (Sigma), Vanilin (Sigma)
X	Xylidine (Sigma)
Y	Yeast extract (Sigma),
Z	Zinc sulfate heptahydrate (Sigma)

[a] the name of the supplier given in brackets: Aldrich - Aldrich Chemical Company, Inc., Milwaukee, WI., USA; BDH - BDH Chemicals Canada Limited, Toronto, ON., Canada; Fisher - Fisher Scientific, Ottawa, ON., Canada; Company, Sigma - SIGMA Chemical Company, St. Louis, MO., USA.

Part III

Results and Discussion

Chapter 7

Enzyme Production

Work described in this chapter was performed because the enzyme preparation used in this study is not commercially available and had to be produced *in situ*. The presented results should be seen as a guideline for a more detailed study on optimization of the production of this enzyme by *Trametes versicolor*.

7.1 Preliminary experiments

During preliminary experiments, a white rot fungus *Trametes versicolor* ATCC 42530 was grown in a liquid medium that was slightly modified from the one used by Khan and Overend [1990]. Bactopeptone, ammonium hydrosulphate and magnesium phosphate used in their medium were replaced by nutrient broth. The Vogel's minerals were also omitted. In the original method the enzyme production required an 18-23 day culture cultivation and the enzyme activity varied between 40.0 and 60.0 nkat per mL of the culture broth. Relatively similar results regarding the time scale of the growth were obtained using the modified medium, but the enzyme activities were 4 times lower than those reported by Khan and Overend [1990]. The changes in the sugar and biomass concentrations, and in the enzyme activity (Figure 7-1) indicated that the enzyme was secreted during the logarithmic and stationary phases of the growth. An increase in the enzyme activity was accompanied by an increase in pH of the medium. The changes in pH of the medium shown in Figure 7-1 represent a typical pH curve observed in all of the performed experiments: an initial phase of a decrease in the pH level, followed by a phase of constant pH, and finally a phase where the pH of the medium was increased steadily. Since the length of each phase depends on the growth conditions, the changes in the pH of the culture broth could be used to monitor the enzyme

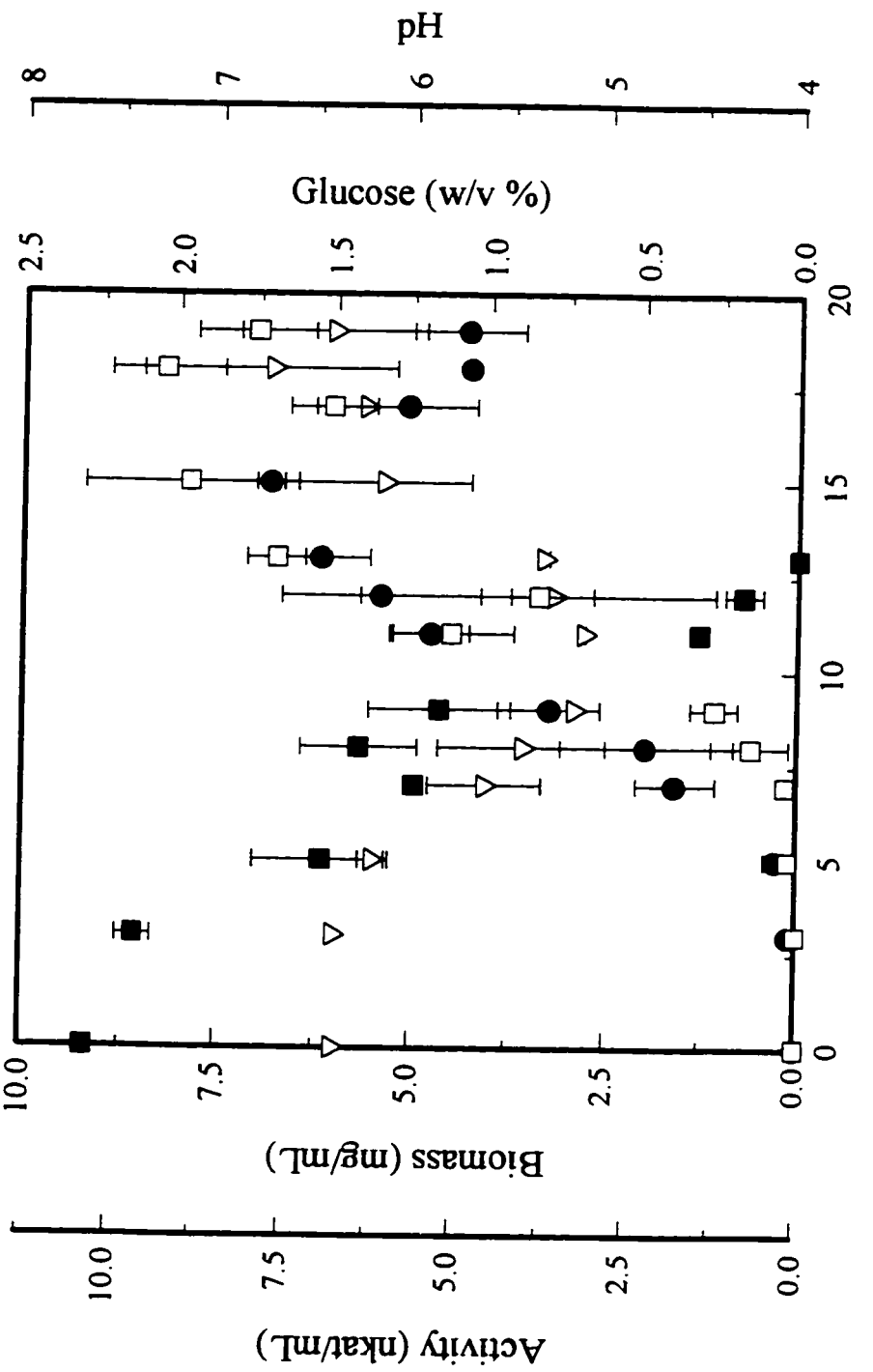


Figure 7-1. Dynamics of the growth of *Trametes versicolor* at 30°C. (▽) -pH; (■) - glucose; (●) - biomass; (□) - enzyme activity.

production, if a correlation between the pH of the medium and the amount of enzyme produced were known.

7.1.1 Method of inoculation

Relatively large variances in the collected data encountered in the already described experiment (Figure 7-1) were caused by an inadequate inoculation procedure that was adopted from the original method. Considering that *T. versicolor* grows in the form of pellets, a sampling procedure to obtain representative data for the culture population is essential. For instance, when a size of the sample taken from the batch is small no reliable data can be acquired. On the other hand, when the changes in the biomass are measured from the entire batch small deviations can be expected. Unfortunately, using the original method of inoculation it was practically impossible to inoculate several batches with the same amount of biomass taken from the agar slant of *T. versicolor*. Bearing this in mind, a special inoculation method, which is described under *Experimental Aspects*, was developed for this work. This method reduced the error in the collected data, because it allowed the inoculation of several batches with virtually equal amounts of inoculum. Consequently, after inoculating following this procedure, the changes in biomass concentration at particular process conditions could be determined from the entire batch, and the effects of system variables on the fungus growth can be studied. Unfortunately, for practical reasons related to the limitations associated with available equipment, it was possible to simultaneously carry out only 24 batch cultures.

7.2 Final experiments

Having established a reliable experimental procedure for inoculation, the next step was to evaluate the possibility of decreasing the time of enzyme production, since from the economical and engineering points of view, a long process time should be avoided. In these experiments, the effects of initial pH of the medium, initial biomass, carbon source and Vogel's minerals concentrations were tested. In one experiment the effect of inducer was also examined.

7.2.1 Effect of Vogel's minerals and pH on enzyme production

To determine whether the absence of the Vogel's minerals was responsible for a lower amount of the produced enzyme, the growth in media with two concentrations of the minerals, and a control were carried out for nine days. At the end of the process the biomass was harvested and the enzyme concentrations in the culture broths were measured. The results (Table 7-1) showed

Table 7-1. Effect of Vogel's minerals on the growth outcome.*

Minerals concentration (%, v/v)	initial pH	Biomass (mg/mL)	Activity (nkat/mL)	Specific productivity (nkat/mg)
0.0	6.4	9.25±0.61	1.44±0.06	0.16
0.2	6.2	8.68±0.35	16.29±1.52	1.87
0.5	5.4	8.29±0.30	13.25±1.13	1.60

* Microbial growth carried out at 30°C; initial glucose concentration 2.3% (w/v), initial biomass 0.09 mg/mL.

that the presence of minerals increased the amount of enzyme at least ten fold. On the other hand, the increase in minerals concentration indicated a possible a decrease in the amount of biomass produced, although the results shown in Table 7-1 were not significantly different at 95% confidence level. Considering that the addition of the minerals changed the initial pH of the medium, it was necessary to determine whether the obtained results were caused by different initial pH levels. To examine this, tests were carried out at 4 different initial pH of the medium supplemented with the same concentration of Vogel's minerals.

The results obtained (Figure 7-2) showed that the initial pH did not affect the production of biomass. The biomass concentrations after 9.0 days of cultivation were 2.28, 2.17, 2.08, and 2.18 mg/mL in the systems with initial pH of 5.2, 5.5, 6.2, and 6.5, respectively. Similarly, the rate of sugar utilization was also not affected by the initial pH. On the other hand, an increase in the pH from 5.5 to 6.5 caused a 30.0% decrease in the enzyme yield. These results showed that the apparent optimum initial pH for the enzyme production should be around 5.5. In light of these results, the discussed earlier effect of the Vogel's minerals (Table 7-1) was less obvious and, therefore, it was studied more extensively.

Five media with different concentrations of minerals but the same initial pH (5.5) were inoculated with 50.0 mg of homogenized biomass each (85 mL final volume of the medium per flask). During these tests it was observed that in the systems with a higher concentration of minerals the number of fungal pellets as well as their sizes were much smaller, suggesting an inhibition of the growth. This observation was consistent with the results regarding the rate of sugar utilization. A decrease in the rate of glucose consumption was observed with an increase in the concentration of Vogel's minerals (Figure 7-3). The tests also showed that the concentration of 0.5% w/v of minerals in the medium was optimum for the production of enzyme. Comparing the

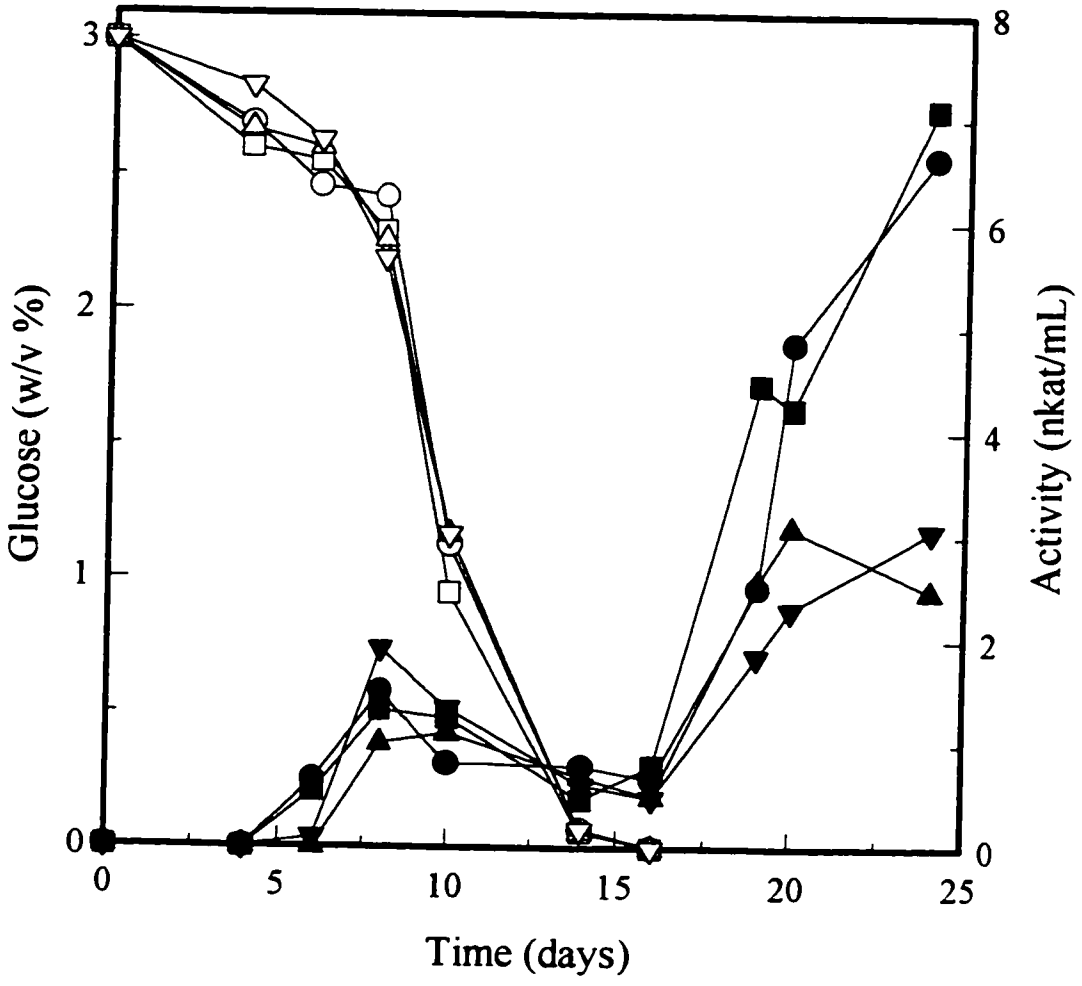


Figure 7-2. Effect of the initial pH of the medium on the glucose utilization and enzyme production: open and solid symbols represent glucose and enzyme concentrations, respectively: (Δ) (▲) pH 6.5; (▽) (▼) pH 6.2; (○) (●) pH 5.5; (□) (■) pH 5.2. Temperature 30°C, initial biomass concentration 5×10^{-3} mg/mL.

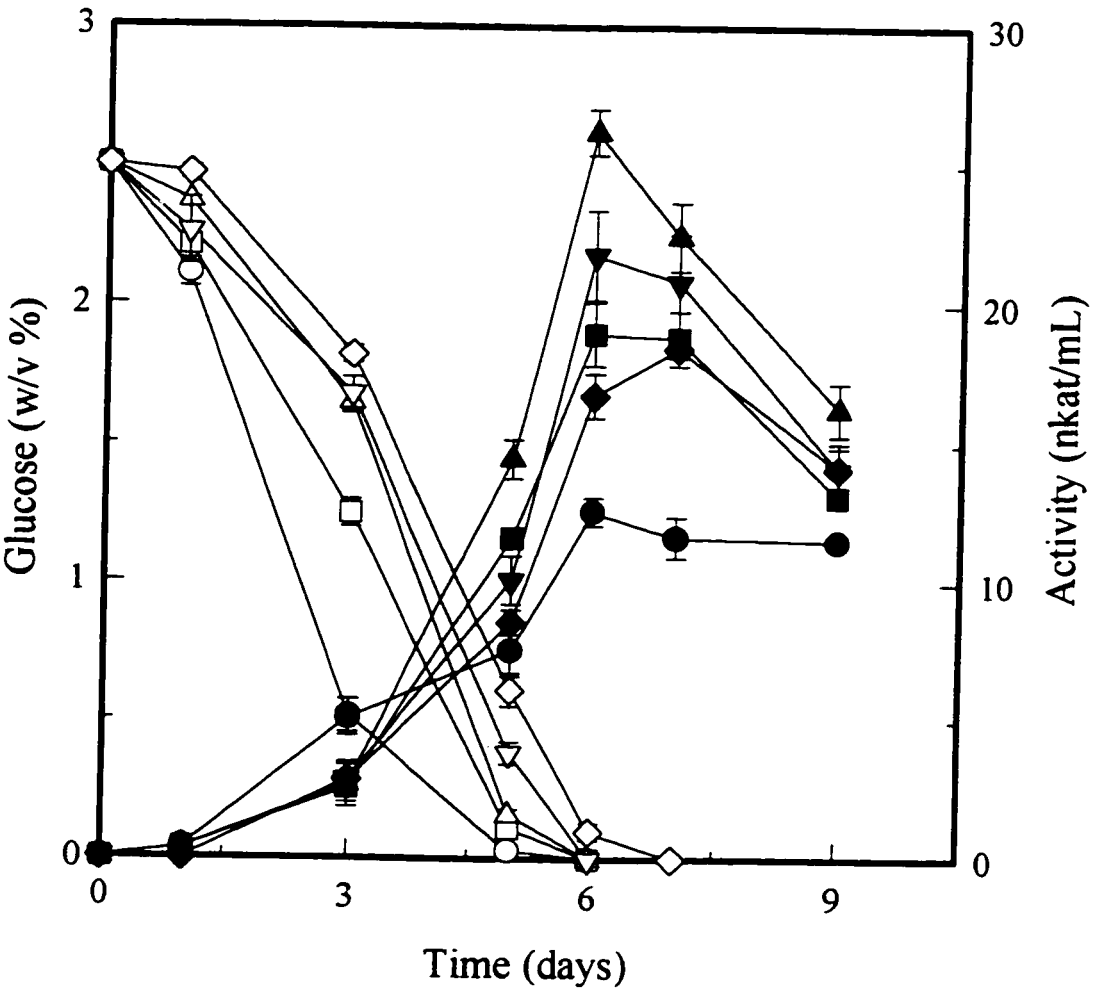


Figure 7-3. Effect of Vogel's minerals on the glucose consumption and the enzyme production: Open and closed symbols represent glucose and enzyme, respectively. (◊) (◆) 1.0%; (▽) (▼) 0.75%; (Δ) (▲) 0.5%; (□) (■) 0.25%; (○) (●) 0.0%. Temperature 30°C, initial pH 5.5, initial biomass 0.59 mg/mL.

enzyme yield obtained at this concentration after the 6th day of the fungal growth with those obtained when the concentrations of minerals were 0.0%, 0.25%, 0.75% and 1.0%, a 52.8%, 27.7%, 16.8% and 36.1% lower enzyme yields were obtained, respectively. Considering these results, in all of the consecutive experiments the medium was supplemented with 0.5% (w/v) Vogel's minerals.

7.2.2 Effect of the inoculum concentration

To investigate the effect of the initial inoculum concentration on the enzyme production, the medium was inoculated with various amounts of the homogenized biomass. The tests with initial inoculum (biomass) concentrations of 0.024, 0.096, 0.24, and 0.48 mg per mL of the medium were carried out. The summary of the changes in the glucose concentration and enzyme activity observed in these experiments is shown in Figure 7-4. As expected, the higher the initial biomass concentration, the faster was the initial sugar utilization. However, its rate was not directly proportional to the amount of inoculum. In the case of the largest inoculum, the glucose concentration decreased to zero after 5 days of growth, while in the system with 20 times smaller inoculum the sugar disappeared after 7 days. A stronger effect of the amount of inoculum was observed during the initial stage of the process, as compared to the later phase where the lines representing the changes in sugar concentration were almost parallel. Considering enzyme production, prior to the 5th day of growth the expected trend was visible, *i.e.*, the higher inoculum the more enzyme was produced. However, after that time the tendency was almost reversed; more enzyme was produced in the system with the inoculum of 0.096 mg/mL than in the two systems with larger inoculum. Neglecting the batch with the smallest inoculum concentration, it can be concluded that the amount of the enzyme produced was reversibly proportional to the initial biomass concentration (Figure 7-4).

The results obtained also showed that a twenty times increase in the amount of inoculum caused the stationary phase to begin 48 hours earlier, but resulted in a 35.0% lower amount of the produced enzyme. Thus, it can be expected that the enzyme production can be accelerated by a higher initial biomass concentration, but the use of a too concentrated inoculum can result in lower enzyme yields.

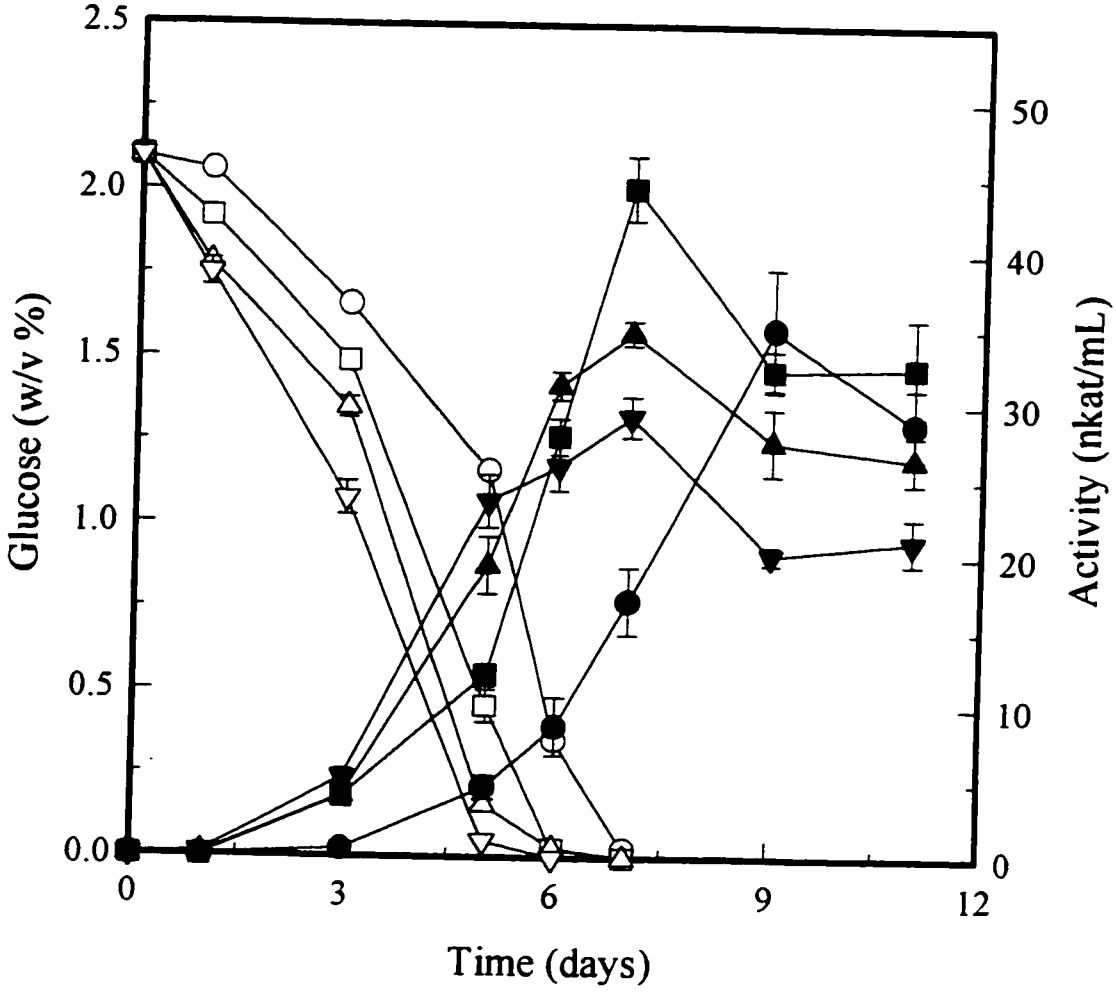


Figure 7-4. Effect of the amount of the inoculum on the changes in the glucose and enzyme concentrations during the growth of *Trametes versicolor*. Closed symbols and open symbols represent glucose and enzyme concentrations, respectively. (○) (●) 0.024 mg/mL; (□) (■) 0.096 mg/mL; (Δ) (▲) 0.24 mg/mL; (▽) (▼) 0.48 mg/mL.

7.2.3 Effect of sugar concentration

To investigate the effect of the initial glucose concentration on the biomass and enzyme production, tests with four initial concentrations of glucose were carried out. The results obtained showed that an increase in the sugar concentration resulted in the production of a greater amount of biomass (Figure 7-5A), but it had no effect on the amount of enzyme produced (Figure 7-5B), except in the case of 0.65% glucose concentration. On the other hand, the increase in the glucose concentration decreased the maximum enzyme productivity. The results also showed (Figure 7-5C) that the rate of sugar utilization was the highest in the batch with 2.36% glucose. When the results for the 5th day of the cultivation were considered (Table 7-2) it was found that the increase in the initial glucose concentration caused a decrease in the enzyme and increase in biomass productivities. Almost 1.5 times lower enzyme and 1.3 times higher biomass productivities were observed in the system with 4.7% initial concentration of the glucose compared to the system with 2.3%. These opposite patterns were obtained because the compared batches were in different stages of the growth cycle. In the system with the lower glucose concentration, the culture was probably already at the end of the logarithmic phase or in the early stationary phase and, therefore, more enzyme was secreted. Considering the results obtained at the moment when the maximum activity was reached, the enzyme volumetric productivity and specific productivity were reversibly proportional to the initial glucose concentration while the biomass yield and its productivity were apparently on the amount of initial sugar.

To compare the enzyme and biomass yields at the same moment of growth and to determine the effect of the initial glucose concentration on the biomass and enzyme productivities at the moment of the total sugar utilization, additional tests were performed. In these experiments the glucose concentrations varied the range from 0.35% to 7.0%. Changes in the sugar concentration were followed in each of the systems until the sugar disappeared. At that moment the biomass was collected and the enzyme activity measured. These results showed (Table 7-3) that the amount of enzyme produced decreased with the decrease in the initial sugar concentration. In the system with 1.1% glucose, the enzyme concentration was 3 times lower than in the system with 7 times higher initial sugar concentration. However, the enzyme yield was 2.25 higher in the system with the lower glucose concentration. On the other hand, when the enzyme productivities were considered, it was found that the optimum glucose concentration to achieve the maximum productivity was 3.0%. As shown in Table 7-3, both enzyme and biomass yields were reversibly

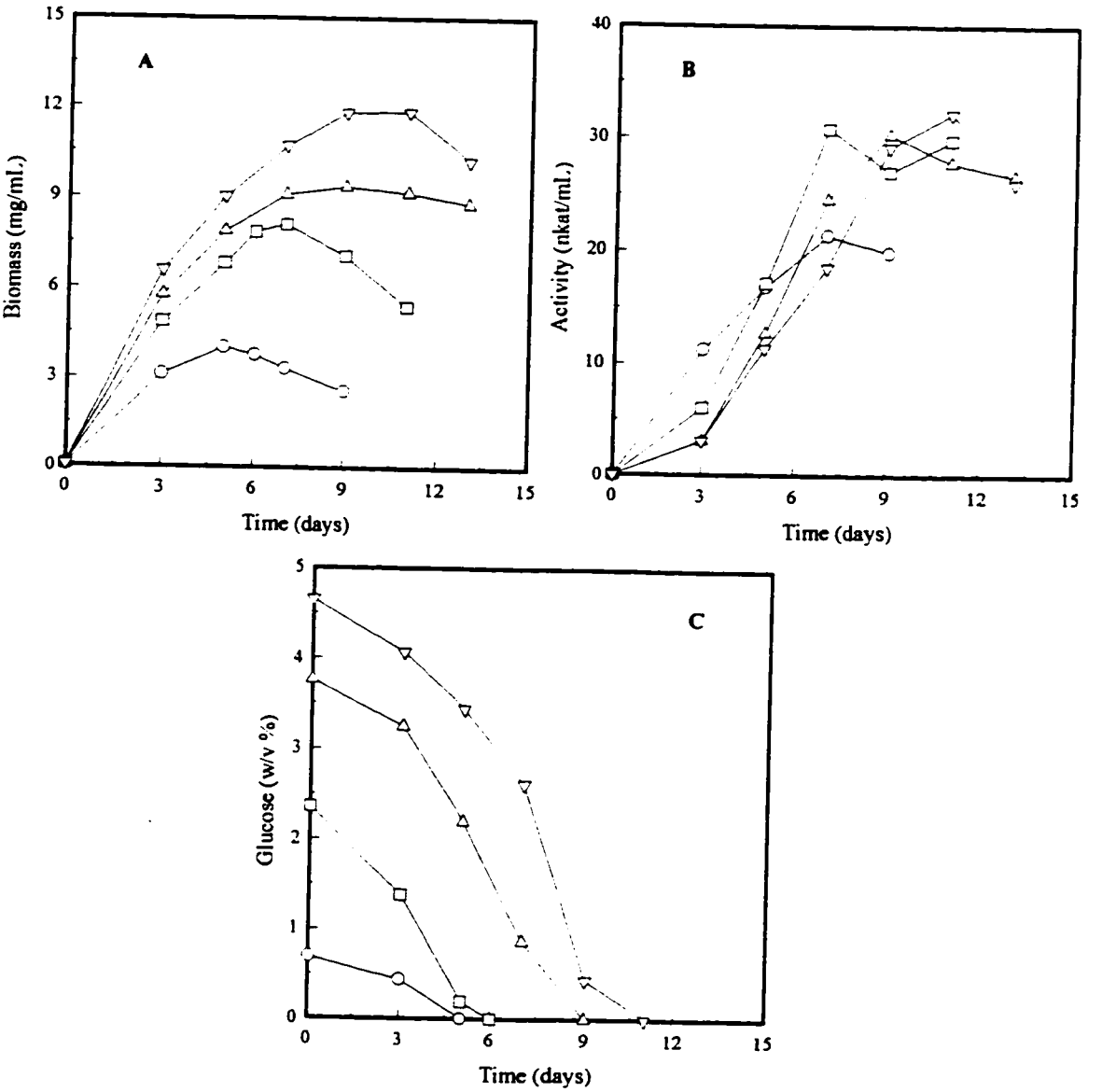


Figure 7-5. Effect of the initial glucose concentration on the biomass production (A); enzyme production (B); glucose utilization (C) during the growth of *T. versicolor* at 30°C. Initial glucose concentration (%, w/v): (O) 0.65; (□) 2.36; (Δ) 3.75; (∇) 4.75. Initial biomass concentration 0.65 mg/mL, initial pH 5.5, initial Vogel's minerals 0.5% (v/v).

Table 7-2. Effect of initial glucose concentration on the biomass and enzyme production at the 5th day of growth, and at the moment of the maximum enzyme activity.*

Time	Initial glucose concentration (% w/v)	Biomass productivity (mg/(mL h))	Enzyme productivity (nkat/(mL h))	Specific productivity (nkat/mg)	Biomass Yield (mg/mg)
5th day	0.65	0.033	0.140	4.24	0.67
	2.36	0.057	0.142	2.51	0.33
	3.75	0.066	0.105	1.60	0.50
	4.75	0.075	0.094	1.25	0.74
7th day	0.65	0.020	0.129	6.45	0.48
7th day	2.36	0.048	0.183	3.81	0.34
9th day	3.75	0.043	0.140	3.25	0.25
11th day	4.75	0.045	0.122	2.71	0.25

* Growth conditions the same as described in the caption of Figure 7-5

proportional to the initial sugar concentration. However, the specific enzyme productivity increased by 27.0% when the glucose concentration increased from 0.35% to 7.0%.

The data presented in Table 7-3 were used to find the correlation between the biomass productivities and the initial sugar concentration. As presented in Figure 7-6 the data could be described using the Eq.(7.1). In general, this equation describes an inhibitory effect of a higher variable value, in this case the substrate concentration, on a given response, in this case the biomass productivity.

Table 7-3. Effect of the initial glucose concentration on the biomass and enzyme production at the moment of the total glucose utilization.*

Initial glucose (% w/v)	Biomass (mg/mL)	Enzyme (nkat/mL)	Yield		Productivity		
			Enzyme (nkat/mg)	Biomass (mg/mg)	Specific (nkat/mg)	Biomass (mg/(mL h))	Enzyme (nkat/(mL h))
0.35	2.82±0.26	3.87±0.62	1.106	0.863	1.37	0.046	0.063
0.60	4.11±0.03	5.92±0.48	0.987	0.685	1.44	0.057	0.082
1.10	5.63±0.36	8.08±0.54	0.734	0.512	1.43	0.058	0.083
3.00	8.52±0.93	17.74±1.56	0.591	0.284	1.85	0.054	0.099
5.00	10.82±0.33	17.78±1.78	0.355	0.216	1.64	0.037	0.060
7.00	12.83±0.96	24.10±5.35	0.344	0.183	1.88	0.035	0.066

* Growth carried out at 30°C, initial biomass concentration 0.27 mg/mL.

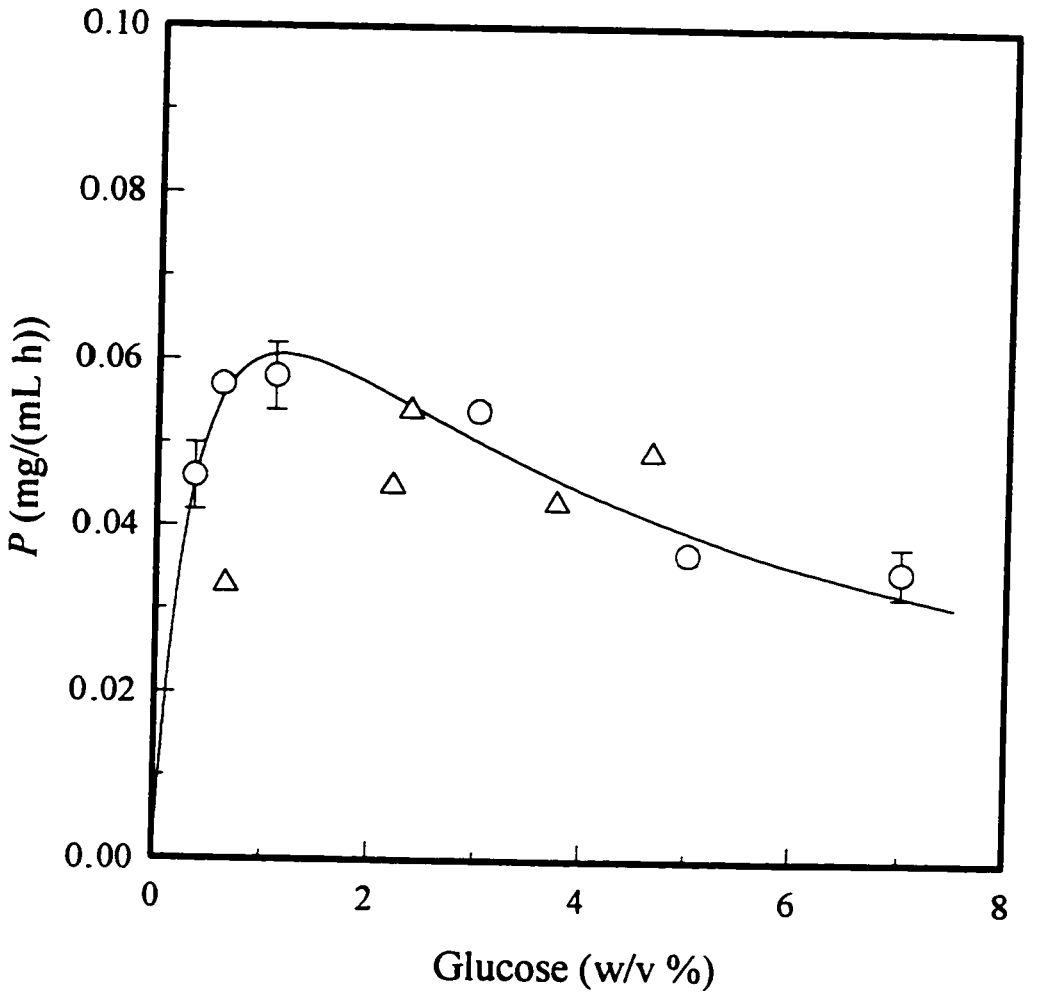


Figure 7-6. Effect of the initial glucose concentration on the biomass productivity at the moment of the total glucose utilization. (O) - data from Table 7-3; (Δ) - data extracted from other growth experiments. Line represents the predicted data using Eq.(7.1).

$$P = \frac{P_{max} S}{S + K_s + K_i S^2} \tag{7.1}$$

where: P_{max} represents maximum productivity if the inhibition is not present, K_s is the substrate concentration at which productivity reaches half of its maximum if the inhibition is not present, and K_i is the inhibition constant.

The data presented in Figure 7-6 were used to obtain the values of the parameters in Eq.(7.1) by the non-linear regression method. The least-squares estimates for the parameters P_{max}, K_s, K_i are 0.095±0.014 mg/(mL h), 3.39±0.12 mg/mL, 5.06±0.07 mL/mg, respectively. Figure 7-6 also shows that additional data extracted from other growth experiments likewise follow the predicted pattern. Using Eq.(7.1) with the above estimates for the respective parameters, it was calculated that the maximum biomass productivity can be achieved when the initial glucose concentration is 1.15% w/v.

7.2.4 Fed-batch versus batch process

Noting that the increase in the glucose concentration suppressed biomass productivity and that specific enzyme productivity was relatively independent of the initial glucose concentration, a fed-batch technique was applied to increase the enzyme production. In these experiments, additional amounts of glucose were successively added after 60, 96, and 120 hours to the medium initially containing 1.26% of glucose. Each time the glucose concentration was increased by 0.5%. The total glucose concentration in the fed-batch experiment corresponded to the initial concentration in the batch system that was analyzed at the same time. The results obtained showed (Figure 7-7) that the amount of enzyme produced using fed-batch technique was greater than in the batch experiment. The rate of its production was also slightly higher. The enzyme yields compared after 9 days of growth varied by more than 40.0% in favour of the fed-batch method. When the biomass was harvested at the end of 14th day of the experiment, it was found that amount of biomass produced was apparently the same in the batch and fed-batch process, 0.70 and 0.68 mg/mL, respectively.

7.2.5 Effect of inducer

In another experiment the effect of an inducer was examined because it has been reported that the addition of an inducer resulted in a threefold increase in the enzyme activity between constitutive and inducible forms of laccase from the same fungi [Leonowicz *et al.*, 1984]. In this

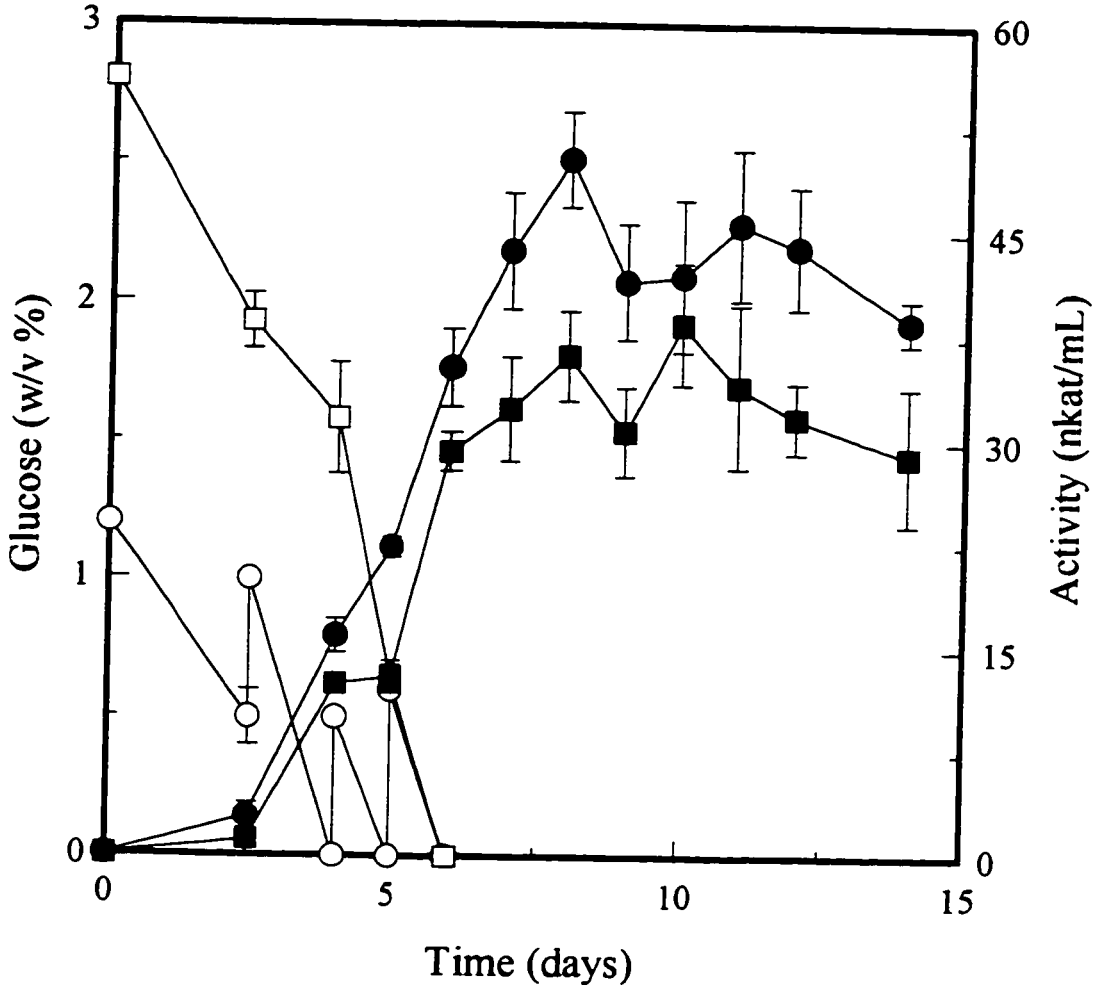


Figure 7-7. Comparison between batch and fed-batch cultures on the enzyme production. (□) - glucose batch; (○) - glucose fed-batch; (■) - enzyme batch; (●) - enzyme fed-batch. Temperature 30°C, initial inoculum 0.27 mg/mL.

experiment 2,5-dimethylaniline (xylidine) was added to a 5 day old culture containing 0.5% Vogel's minerals. Xylidine is a known inducer for the production of laccase from *T. versicolor* [Fåhraeus and Reinhamard, 1967, Leonowicz *et al.*, 1984; Rogalski *et al.*, 1991], and the 5th day induction is considered the optimum for this fungus [Rogalski *et al.*, 1991]. The obtained results are shown in Figure 7-8. As judged from the changes in the concentration of glucose after the 5th day, the addition of inducer caused a slight inhibition of the growth. On the other hand, the measured activities were almost three times higher in the induced culture than in non-induced one (Figure 7-8). The both enzyme preparations caused the same changes in the scanwavelength of ferulic acid. This result could indicate that both preparations could have similar properties.

However, considering that the non-inducible form of the enzyme caused the cleavage of the aromatic ring in phenolic compounds [Khan and Overend, 1990] this enzyme was used in the other part of this study.

7.2.6 Summary of enzyme production

Analyzing the effects of the initial process variables on the growth of *T. versicolor*, it was found that the enzyme production required the presence of the Vogel's minerals and depends on the initial pH of the medium. The increase in the initial biomass concentration accelerated its production. However, the optimum amount of inoculum should be used to obtain the greatest enzyme yield.

Considering these results, the non-induced enzyme preparation used to decrease the phenolic content in canola meal was produced using the following conditions: the initial concentrations of glucose, minerals, nutrient broth, and yeast extract were 2.0%, 0.5%, 0.5%, and 0.8% (w/v), respectively. The initial pH of the medium was 5.4. The medium was inoculated with the homogenized 6-7 days old biomass, in the amount resulting in the 0.080 mg of biomass per mL of the medium. The process was carried out at 30°C for 8 days. The filter sterilized culture broth, without additional purification, containing 40 nkat/mL of ferulic acid oxidase, was divided into several small batches and was stored frozen until used as the enzyme preparation for the experiments in the model and CM systems.

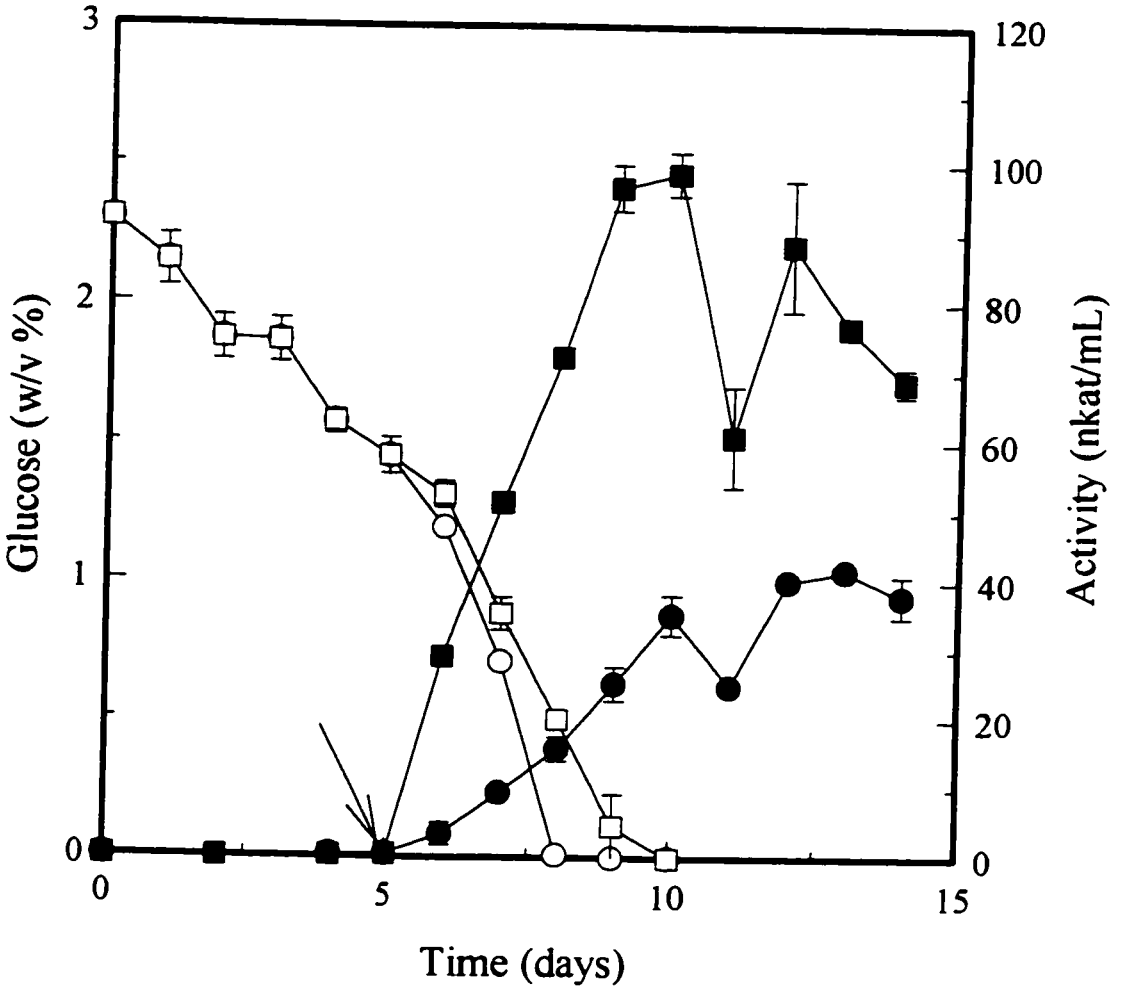


Figure 7-8. Effect of the addition of the inducer (xylidine) on the enzyme production and glucose utilization. Arrow indicates addition of the inducer. Non-induced culture: (O) - glucose; (●) - enzyme. Induced culture: (□) - glucose; (■) - enzyme. Temperature 30°C, initial biomass 0.016 mg/mL.

Chapter 8

Model Enzymatic System

8.1 Enzyme preparation characteristics

The enzyme preparation used in this study was characterized in a model system containing one of the following substrates: sinapic acid (SA), sinapyl alcohol (SAC), sinapaldehyde (SALD), and sinapine bisulfate (SIN). The analysis included investigation of the effects of temperature, pH, substrate and enzyme concentrations, and presence of minerals. The thermal stability of the enzyme was also investigated. The results from this part of the project served as a guideline for the development of the enzymatic process for the reduction of phenolics content in canola meal.

8.1.1 Enzymatic transformation of sinapic acid and its derivatives

Figure 8-1 displays the changes in ultra-violet and visible absorption of the tested substrates after the addition of the enzyme. The gradual loss of absorbency of SA at 310 nm, of SALD at 340 nm, and of SIN at 328 nm with time indicates that the enzyme preparation transformed these compounds. The lack of appearance of any secondary absorbency indicates that the measurements of the changes in absorbency at these wavelengths should be used for the determination of initial reaction rates for these compounds. It should be noted that the activities evaluated using this technique are underestimated, due to the overestimation of the substrate concentration [Łącki and Duvnjak, 1996a]. The latter is caused by the reaction products which increase the absorbance at the wavelength being used for measurements. The comparison of the

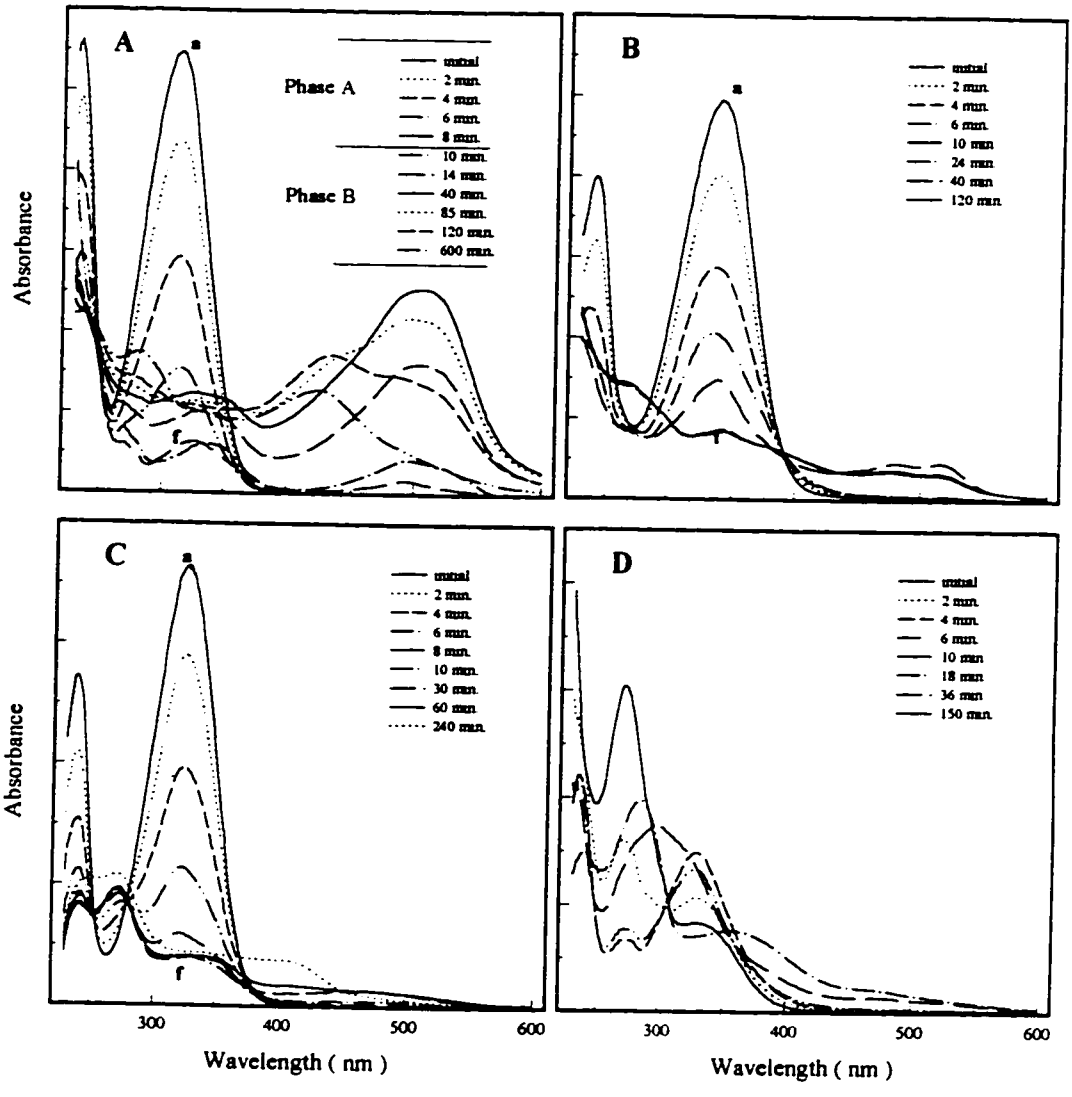


Figure 8-1. Changes in absorbance during the enzymatic conversion of: (A) sinapic acid; (B) sinap aldehyde; (C) sinapine; (D) sinapyl alcohol.

initial and final scanwavelength of the reacting system (Figure 8-1: curves (a) and (f)) indicates that these products cannot give higher absorbance than the corresponding absorbency of the 10.0%, 16.5% and 10.0% of the initial solutions of SA, SIN, and SALD, respectively. Therefore, at any time of the reaction, the concentration of the substrate is overestimated by less than 10.0-16.5% of the substrate transformed [Łącki and Duvnjak, 1996a]. Sinapyl alcohol was also transformed by this enzyme (Figure 8-1D); however, the interference of the reaction products was much larger than in the previous three cases, and the changes in the shape of the scan did not allow the use of the spectrophotometric technique for the evaluation of the enzyme activities without making large errors. This interference and a very low solubility of sinapyl alcohol were the reasons that this compound was not further investigated in this study.

Figure 8-1 also shows that all of the investigated enzymatic transformations took place in at least two distinct stages, Phase A and B. The beginning and end of Phase A is marked by curves (a) and (f), respectively. The end of Phase A marked the beginning of Phase B. On the other hand, the end of Phase B is not very well defined, and, this is the reason why only these two phases of tested transformations are recognized in this work.

8.1.2 Effect of pH on enzyme activity

The pH optima for the reaction systems with SA, SALD and SIN are 4.0, 3.3 and 4.5, respectively (Figure 8-2). The optimum for SA is comparable to that reported for inducible form of laccase produced by this fungus [Rogalski *et al.*, 1990]. A quantitative comparison of the activities was not possible since the conditions for the enzymatic assay were not reported. It was noticed in the present study that the activities for all the substrates were negligible at pH 6.5.

To determine the effect of buffer type on the enzymatic activity, the tests were performed with SIN as the substrate using two additional buffers, glycine and acetate. The results obtained (Figure 8-3) showed that the highest activity was measured in the acetate buffer at pH 3.7. On the other hand, the enzyme was more active in the citrate-phosphate buffer at higher pH levels. The activities measured in the glycine buffer were lower than those obtained using the other buffers. Furthermore, the optimum pH was not defined in the case of glycine buffer, since this buffer covers only a narrow range of pHs.

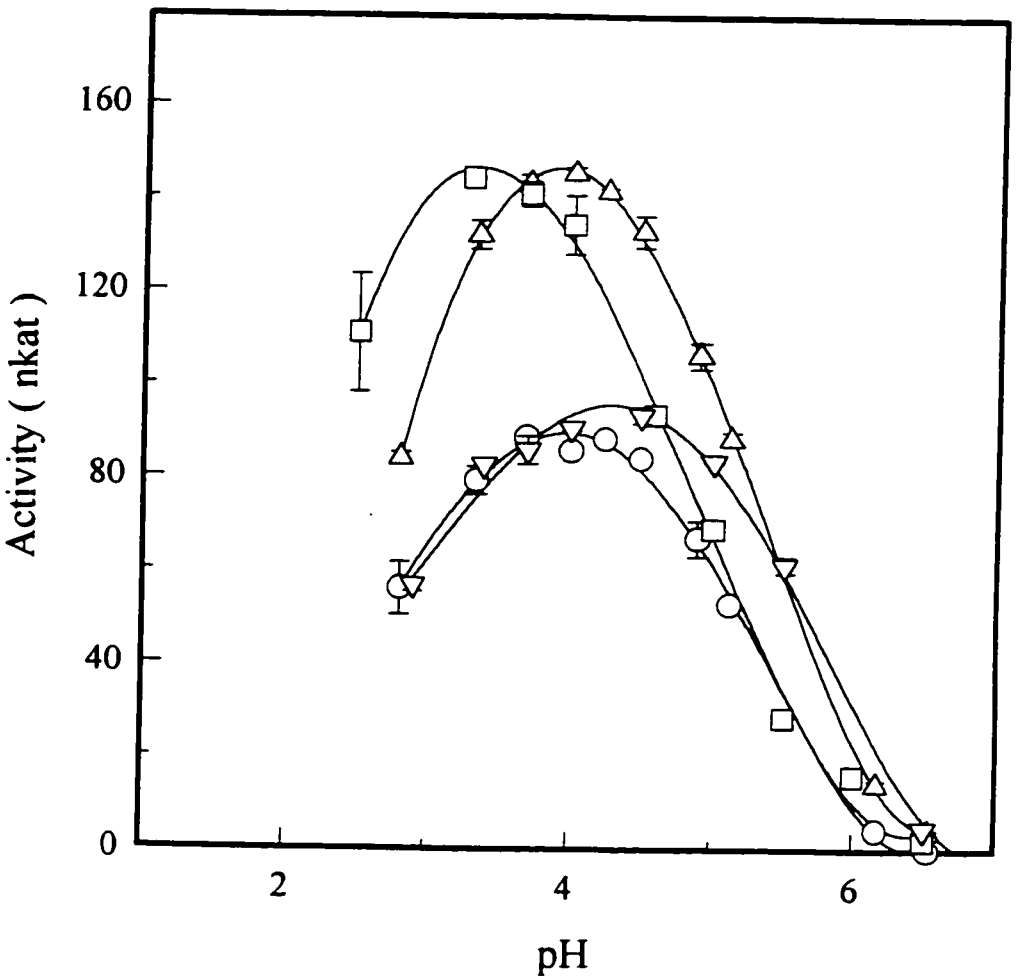


Figure 8-2. Effect of pH on the enzymatic conversion of SA (○) 30°C, (Δ) 50°C; SIN (▽) 50°C; SALD (□) 50°C.

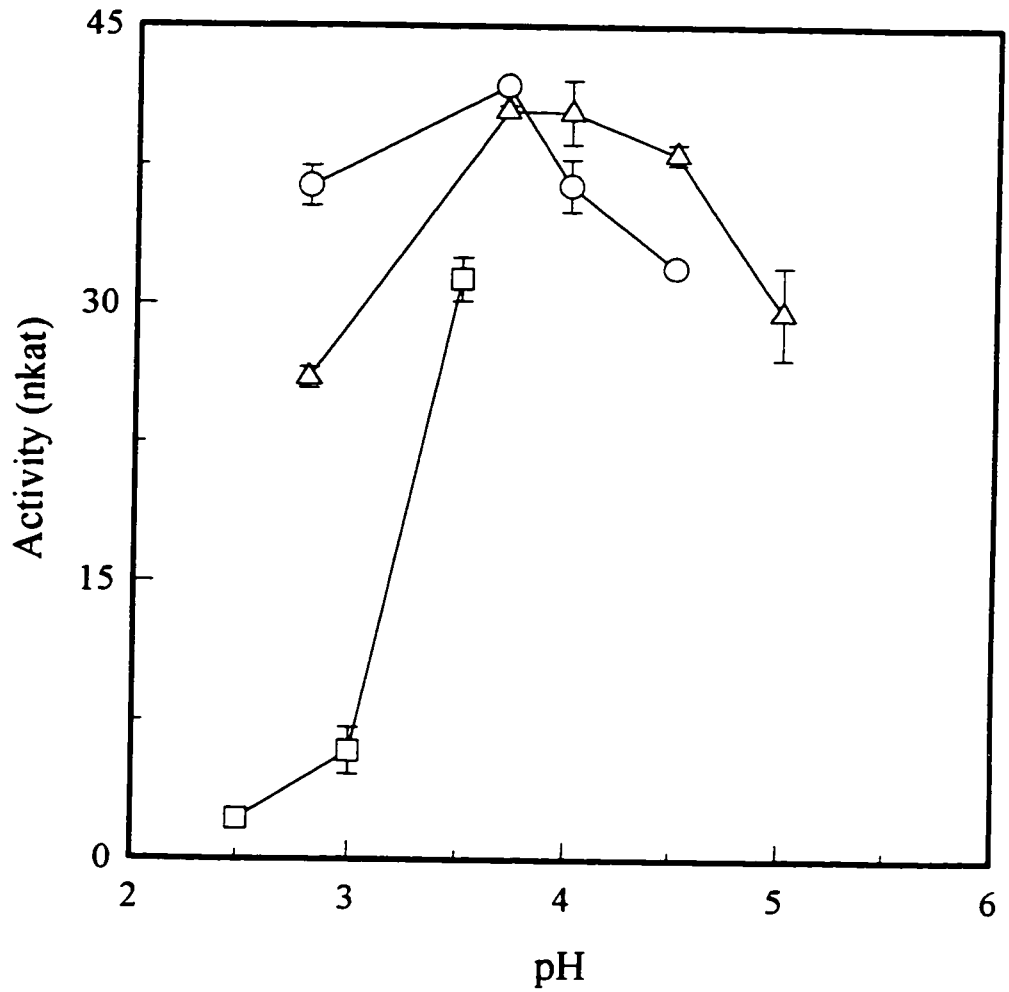


Figure 8-3. Effect of the buffer type on the enzyme activity: (Δ) - citrate-phosphate; (□) - glycine; (○) - acetate.

8.1.3 Effect of temperature on enzyme activity

To determine the optimum temperature for the investigated transformations, the reactions were carried out at various temperatures, ranging from 10°C to 80°C, and at various pH levels. The results obtained (Figure 8-4) show that the optimum temperature for SA conversion measured at three different pH levels is 60°C, while for SALD and SIN transformations is 50°C. The other pH levels were also investigated for the latter substrates. The results regarding SIN will be covered later in the text, whereas the results regarding SALD followed the pattern shown in Figure 8-4 and are not presented for the sake of clarity of the graphical representation.

8.1.4 Effect of substrate concentration

The effect of the substrate concentration on the enzyme activity is shown in Figure 8-5. The inhibitory effect of substrate was observed for SIN and SA. A lack of inhibition by the higher SALD concentration could indicate that steric effects, related to the size of the aliphatic chain, play a role in the enzyme-substrate complex formation [Łącki and Duvnjak, 1996a]. When the first parts of the curves presented in Figure 8-5 were considered, the K_m values for SA, SALD and SIN of 0.024 mM, 0.046 mM and, 0.117 mM were obtained, respectively.

8.1.5 Effect of temperature on Michaelis-Menten constant

The effect of the SA concentration on the enzyme activity at various temperatures is presented in the form of Hanes-Woolf plots (Figure 8-6). It appears that an increase in temperature increases the turnover number, and the K_m values decrease from 0.024 mM at 20°C to 0.016 mM at 50°C. This indicates that the affinity of the enzyme towards sinapic acid increases with temperature. The effect of temperature on the K_m is in agreement with the results reported for pepsin [Bailey and Ollis, 1986]. The heat of the formation of the enzyme-sinapic acid complex and the entropy change, calculated using values of K_m and respective temperatures, are -2557.6 J mol^{-1} and -55.9 J $mol^{-1}K^{-1}$ respectively. However, considering that oxygen is the second substrate for this enzyme, and that its concentration varies with temperature during the measurements of enzyme activity, the K_m values should be considered as apparent ones. Therefore, although it may be that

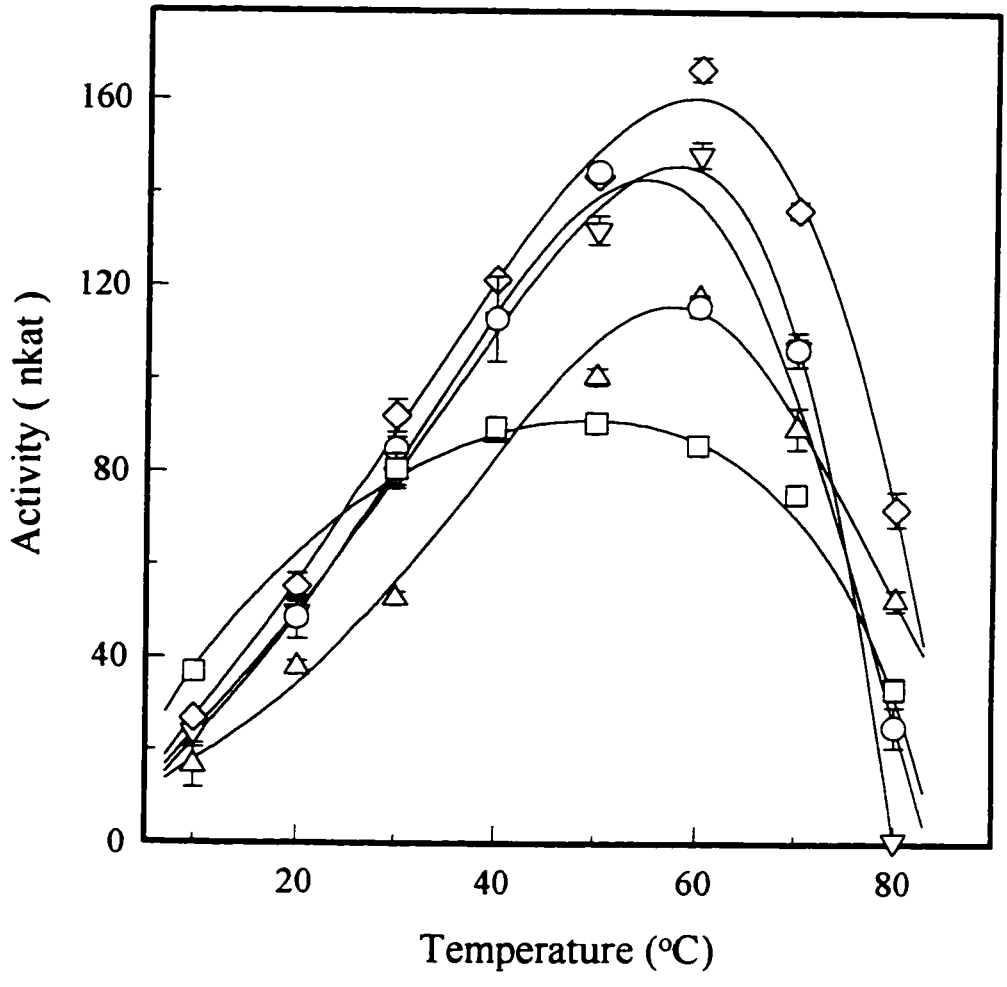


Figure 8-4. Effect of temperature on the enzymatic conversion of SA (∇) pH 3.4, (◊) pH 4.0, (Δ) pH 5.1; SIN (□) pH 4.0; and SALD (○) pH 3.3.

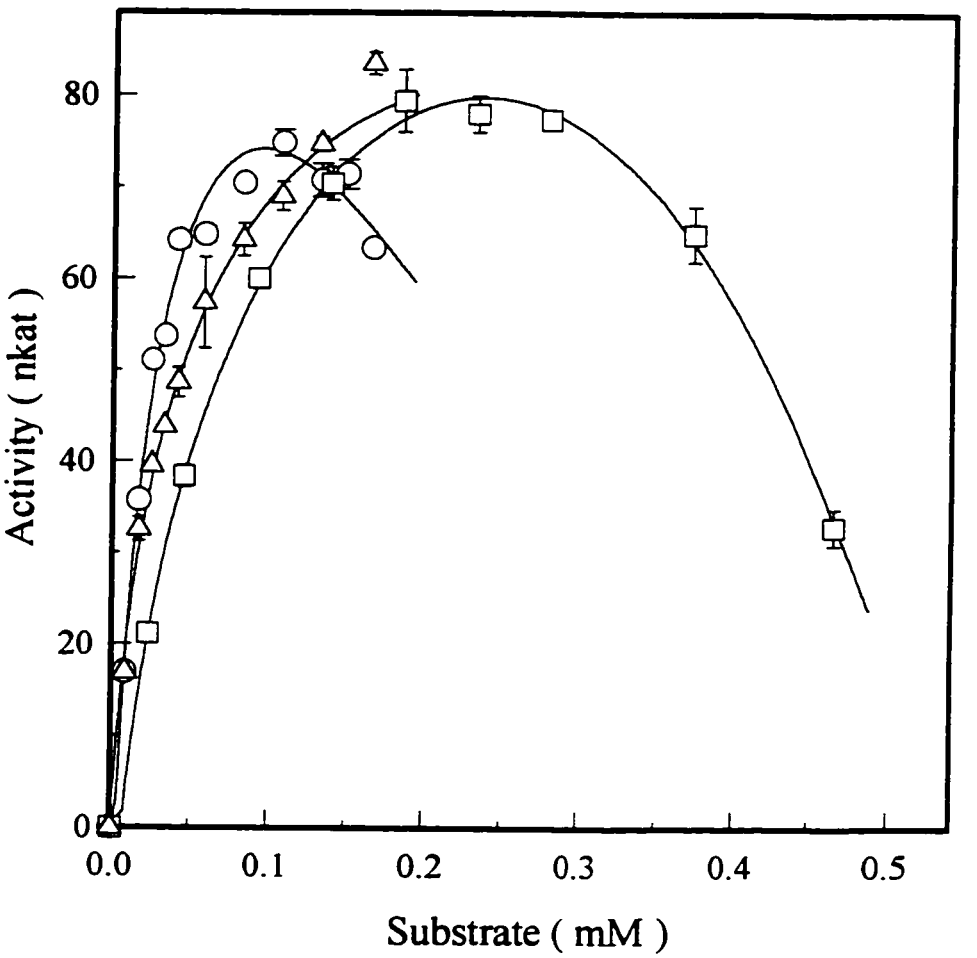


Figure 8-5. Effect of substrate concentration on the enzymatic conversion of SA (O), SIN (□), and SALD (Δ).

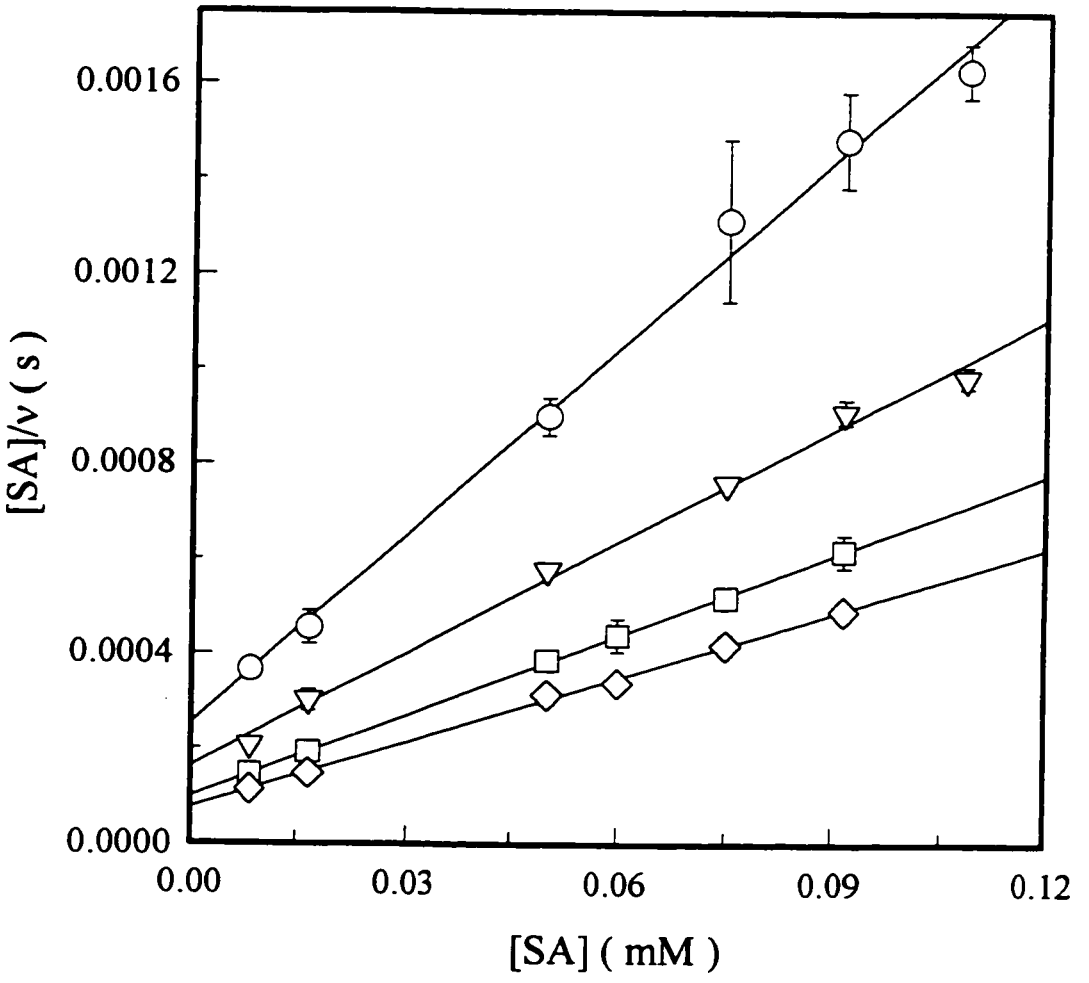


Figure 8-6. Hanes-Woolf plot for the enzymatic conversion of sinapic acid at: (○) 20°C; (▽) 30°C; (□) 40°C; and (◊) 50°C.

temperature does not affect the enzyme affinity towards sinapic acid, the K_m values would differ from the above mentioned if all experiments were performed either at a constant oxygen concentration, or if oxygen concentration was not a limiting factor. The information regarding the saturation of the enzyme with oxygen is rarely reported, probably because of practical difficulties in experimentation, and therefore the activities measured at different temperatures may not provide information about the true temperature optimum. It could be expected that when the enzyme is saturated with oxygen or when running the tests at a constant oxygen level, the optimum temperature would be higher than that when the saturation conditions are not met, and furthermore, the relative ratios of activities at various temperatures under the above two conditions would not hold [Łącki and Duvnjak, 1996a]. This phenomenon is explained in Appendix A.

8.1.6 Enzyme stability at various temperatures and pH levels

The effects of temperature and pH on the stability of the enzyme preparation were investigated. The kinetics of enzyme deactivation at various temperatures are shown in Figure 8-7. A relatively strong drop in the initial activity at 35°C is consistent with 18.2 % deactivation reported after 24 hours of the incubation of inducible form of laccase [Rogalski *et al.*, 1990]. Although boiling of the enzyme for 2 min resulted in a complete loss of activity, the 20% remaining activity still observed after 1.5 hours of preincubation at 70°C indicates a high thermostability of this enzyme.

This thermal deactivation process is well described by Eq.(8.1) [Łącki and Duvnjak, 1996a].

$$\frac{e(t)}{e_0} = \left(1 - x_1\right)e^{-\lambda_2 t} + x_1 e^{-\lambda_1 t} \tag{8.1}$$

where: e_0 is the initial concentration of the enzyme active form expressed in percentages (100%) and $e(t)$ is enzyme concentration after time t (h), x_1, λ_1, and λ_2 are the model parameters.

The estimated values of the parameters in this equation are given in Table 8-1. Although from the mechanistic point of view this equation can describe several deactivation mechanisms, in this study it represents an irreversible deactivation process. This mechanism was chosen

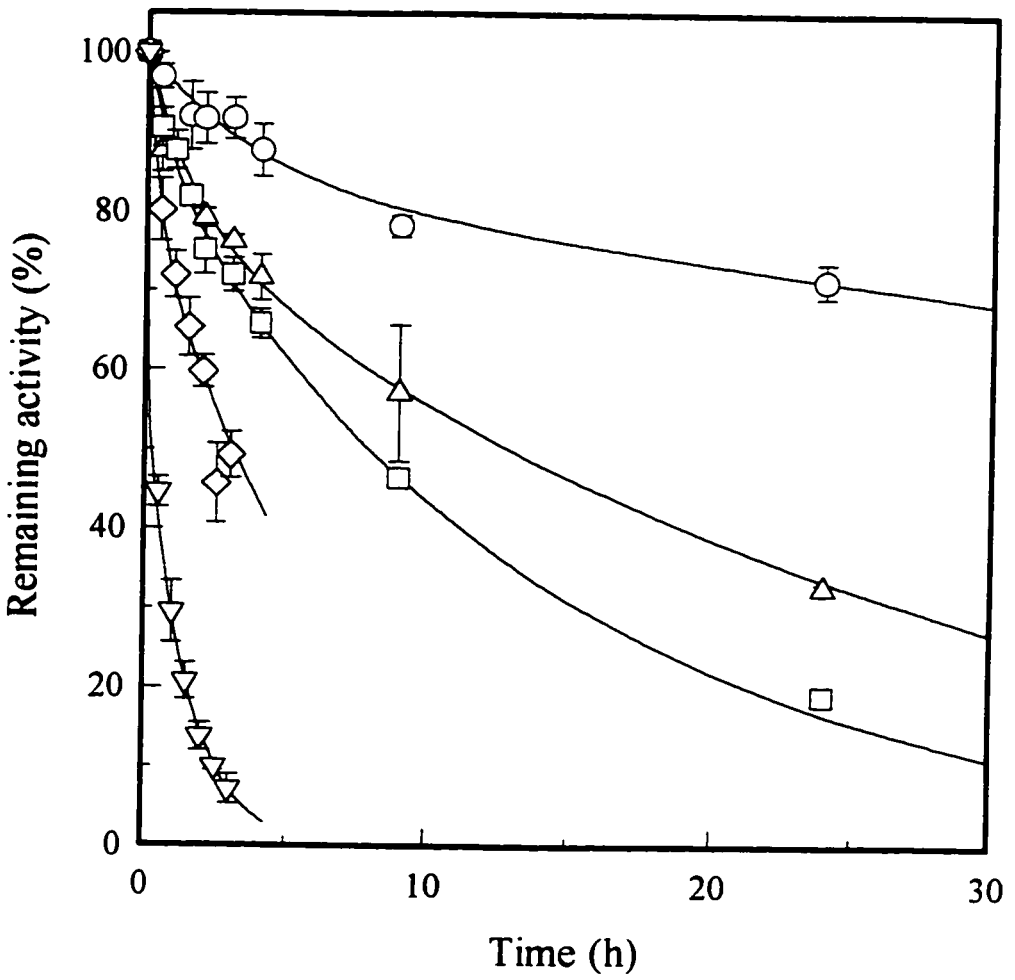


Figure 8-7. Effect of the preincubation time on enzyme stability at: (○) 35°C; (Δ) 45°C; (□) 50°C; (◊) 60°C; and (▽) 70°C. Solid lines as predicted by Eq.(8.1).

Table 8-1. Values of the parameter estimates and their standard deviations

Parameter	Temperature (K)				
	308.2	318.2	323.2	333.2	343.2
x_1	83.98 (1.22)	79.83 (0.99)	86.03 (1.75)	80.13 (1.79)	40.92 (3.47)
λ_1	0.0067 (0.0008)	0.036 (0.001)	0.068 (0.004)	0.153 (0.012)	0.695 (0.075)
λ_2	0.266 (0.052)	0.605 (0.098)	0.974 (0.369)	2.325 (0.681)	18.252 (6.613)

because the tests of cooling of the enzyme preparation at 5°C for shorter or longer periods of time after its preincubation at 50°C failed to restore the initial activity.

The supposed mechanism of enzyme deactivation is identical to the one proposed by Wright and Schoemaker [1949] for inactivation of diphtheria antitoxin. This model assumes that upon heating, the native active form of enzyme (E) can transform either irreversibly to the inactive form (I), and/or reversibly to the so-called protected form (E^p). The scheme for this deactivation mechanism is shown in Figure 8-8, while the derivation of Eq.(8.1) is presented in Appendix B.

$$E^p \underset{k_2}{\overset{k_3}{\rightleftharpoons}} E \xrightarrow{k_1} I$$

Figure 8-8. Mechanism for the thermal deactivation of the enzyme.

The values of the respective kinetics constants (Figure 8-8) calculated from the parameters given in Table 8-1 make it possible to calculate the energy of activation for each of the reactions involved. This was done using the definition of the kinetic constant based on the transition state theory [Bailey and Ollis, 1986] and the nonlinear least-square estimation technique. It was found that the energies of activation for steps 1, 2, and 3 were 160.0 kJ mol^{-1}, 119.668 kJ mol^{-1} and 61.721 kJ mol^{-1}, respectively.

The high thermal stability of the enzyme is also presented in Figure 8-9. The enzyme is the most stable within a pH range of 4.5 and 6.5 regardless of the temperature of preincubation. Some of the results displayed also showed that the enzyme activities were higher upon preincubation at

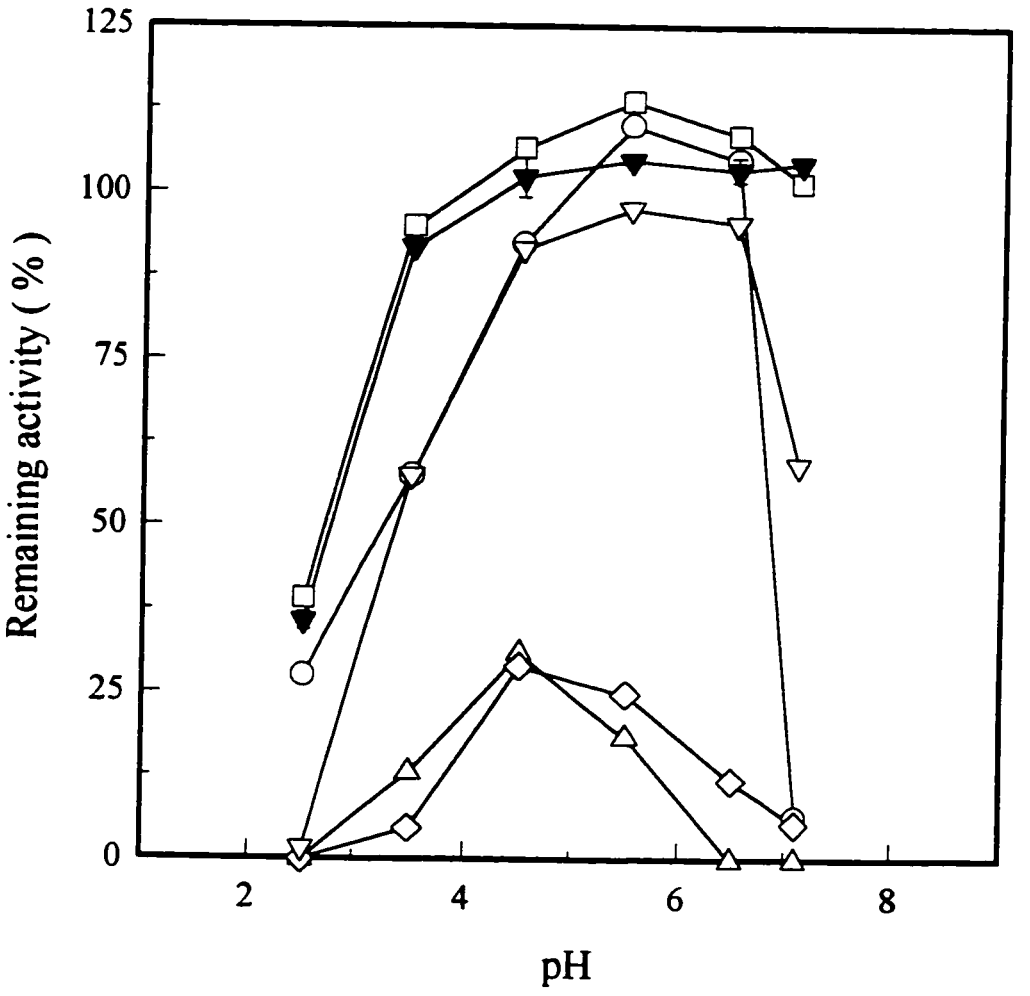


Figure 8-9. Effect of the temperature and time of preincubation of the enzyme at various pH values on its activity: (○) 80°C, 0.5 min; (Δ) 80°C, 6 min; (□) 65°C, 1 min; (◊) 65°C 3 h; (▼) 50°C, 10 min; (▽) 50°C, 3 h.

higher temperatures for a short period of time than without preincubation. The reason for this was that the enzyme preparation used in this set of tests was kept frozen prior to utilization, and it was noticed that the freezing and thawing steps resulted in a partial loss of its activity. Thus, the higher activities upon preincubation represent the restoration of the activity lost due to freezing and thawing. Similar results regarding freezing and thawing have already been reported. Rogalski *et al.* [1990] showed that 10 subsequent freezing and thawing processes resulted in a 61.0% loss in the activity of the inducible laccase, with a 10.0 % drop after the first one.

8.1.7 Effect of inhibitors

The effects of inhibitors on the conversion of sinapic acid by this enzyme are compared with those already reported for transformation of phenolic substrates with enzymes secreted by *T. versicolor* (Table 8-2). It was noticed that sodium azide is the strongest inhibitor. This salt is also a well-known inhibitor of laccases [Reinhammar and Malmstrom, 1981]. The lack of any inhibition by the EDTA, which is opposite to the results reported for the inducible form of laccase [Leonowicz *et al.*, 1984], can be explained by the strong bonds between the metal ions and the enzyme molecule [Omura, 1961]. The inhibition observed with thiocyanate is consistent with the order of inhibitory effectiveness on copper containing enzymes [Reinhammar and Malmstrom, 1981].

Although the results obtained showed that this enzyme could be either a constitutive laccase or a polyphenol oxidase, its reaction with p-cresol (the test used for differentiation between the two enzymes [Butt, 1980]), categorizes it as polyphenol oxidase [Łącki and Duvnjak, 1996a].

8.2 Products of the enzymatic transformation of SA and SIN

Initially, several tests using various separation techniques were performed to determine the products of the enzymatic transformation of sinapine (SIN). These trials were not successful because the product's concentrations were very low and/or techniques used were not suitable to detect these new compounds. For this reason, the development of a specific method for the separation of the products from SIN transformation and their further identification was not considered. On the other hand, the changes in the scanwavelengths of SIN and sinapic acid (SA)

Table 8-2 Effects of inhibitors on the polyphenol oxidase from fungus *Trametes versicolor*

Inhibitor	Conc. [mM]	Inhibition [%]		
		this work	reported	
sodium azide[a]	0.01	-	55.0	[Bollag and Leonowicz, 1984]
	0.10	98.0	100.0	
	2.00	100.0	-	
EDTA[b]	0.1	0.0	6.0	
	2.0	0.0	42.0	
	3.0	0.0	54.0	
potassium thyocyanate[a]	0.01	50.5		-
	2.00	80.1		
	3.00	96.1		
methanol[c]	2.4×10^3	39.7	-	[Leonowicz and Grzywanowicz, 1981]
	11.9×10^3	93.0	50.0	
	16.6×10^3	96.2	-	
hydrogen peroxide[a]	0.002	1.0	-	[Khan and Overend, 1990]
	0.040	6.9	-	
	0.200	23.5	9.1	

[a] added to the mixture of sinapic acid and buffer prior to the addition of enzyme;

[b] enzyme solution preincubated with EDTA for 15 minutes before adding SA;

[c] assay conducted in the respective MeOH concentrations in citrate-phosphate buffer solutions with pH adjusted by citric acid.

recorded during Phase A of their enzymatic transformations (Figure 8-1) showed some resemblance to each other indicating the possible formation of similar products. Therefore, bearing in mind that SIN is the choline ester of SA, it was decided to develop procedures for the separation and identification of the reaction products using commercially available SA. The ultimate goal of this approach was to compare the products from the enzymatic transformation of SA with the products formed during the SIN transformation that could be separated by applying similar procedures to those developed for SA.

8.2.1 Extraction of products

The products formed during the enzymatic transformation of SA were extracted from the reaction mixture using methylene chloride (MeCl). The separation of the organic and water phases ensured termination of the enzymatic reactions that involve compounds extractable with MeCl. MeCl had no effect on extracted compounds. The composition of the concentrated MeCl extracts did not change during 3 months storage.

The effectiveness of the MeCl extraction of the products formed depended on the extent of the enzymatic transformation. For example, a mass balance over the reacting solution done at the moment of disappearance of SA showed that 92.0% of SA was transformed to the MeCl extractable products, whereas 8% accounted for only water soluble compounds. On the other hand, when a mass balance was performed after 24 hours of incubation, it showed that 23.0% of SA was transformed to MeCl extractable compounds, 29.0% formed a precipitate insoluble in water and in MeCl, whereas around 48.0% was transformed to only water soluble products. Extraction with MeCl could be enhanced under acidic conditions (pH 1.0), however, the low pH caused changes in the colour of the reacting solution indicating that new non-enzymatic reactions were taking place. These new reactions lead to the formation of different products that were not part of the actual transformation pathway for the enzymatic oxidation of SA. For the above reasons and because, as will be described later, all of the products formed during Phase A of the SA transformation were extracted with MeCl, only the new compounds extractable with this organic solvent were considered in this work.

8.2.2 Separation of products

The products extracted with MeCl were separated using HPLC. A detailed description of the separation method developed in this work is given under the *Experimental aspects*. This method gave a very good separation of the products regardless of the extent of reaction.

The HPLC method for the separation of the products was developed using extract from a one hour-long transformation of SA. More than 14 products were detected when using this method (Figure 8-10). The HPLC separation data and UV-VIS data (obtained using the PDA detector) for these compounds are given in Appendix E: Table E-1. Eight products, compounds I, II, VII, VIII, IX, XI, XII, and XIV, were collected as fractions in the effluent of the HPLC column during the separation of 1 mL (50 separations, 20μL each) of a MeCl extract obtained by extracting a 3 hour old reaction mixture. These fractions were used for specific analysis to identify the respective products.

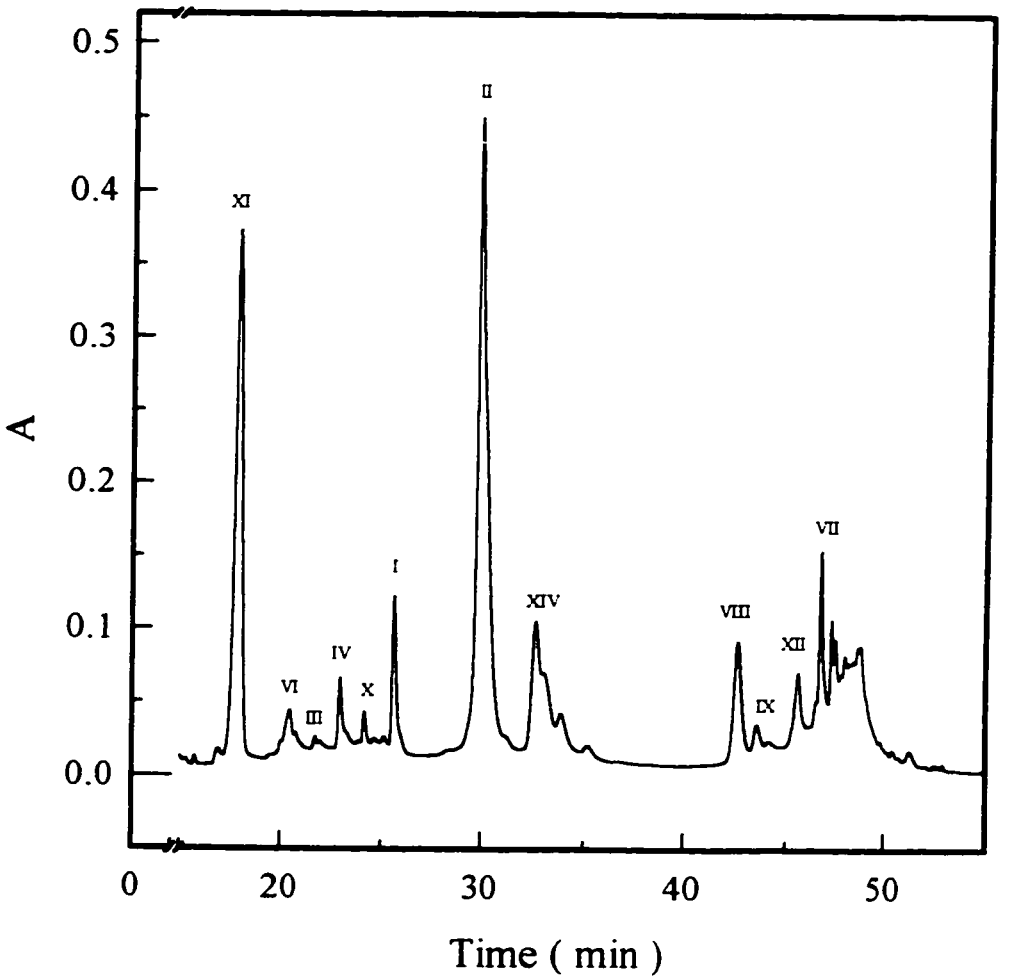


Figure 8-10. Chromatogram of the methylene chloride extract of the products from enzymatic transformation of SA. Detection wavelength: 280 nm.

8.2.3 Dynamics of SA transformation

The dynamics of SA transformation were studied by following the changes in the concentration of the MeCl-extractable products with time. The initial stages of the reaction, up to 1 hour, were examined in more detail. In these experiments the enzymatic reactions were carried out as batch processes, and the products were extracted from the entire batch after various reaction times. The results obtained showed (Figure 8-11) that the enzymatic transformation of SA is a multi-step reaction system. Furthermore, the results confirmed the presence of at least two distinct stages of this overall reaction, as postulated earlier in the text (section 8.1.1). The moment of nearly complete utilization of SA can be considered as the end of Phase A and the beginning of Phase B.

Undoubtedly, Phase A of SA transformation, marked by the formation of compounds I and II and the disappearance of SA, was caused by the enzymatic step (Figure 8-11b). Under the conditions of this experiment, compounds I and II were the predominant products formed in phase A (Figure 8-11(b)-(d)). In fact, their scanwavelengths (Appendix E: Table E-1) were almost identical to the scanwavelength of the enzymatically transformed SA solution at the end of Phase A (Figure 8-1). During this phase, the reacting solution remained colourless.

The disappearance of SA seemed to trigger further reaction steps (Figure 8-11e) responsible for Phase B of SA transformation. The changes in the colour of the reacting solution (dark red appearance) caused by these new reaction steps were similar to those observed during Phase B of SA transformation (Figure 8-1). As shown in Figure 8-11, these changes had to be associated with the transformations of I and II, and the formation of new products, including compounds IV, IX, X, XI, XII and XIV. In particular, the red color (absorbance maximum at 500-550 nm) shown in Figure 8-1 of the reacting solution observed during Phase B was associated with a presence VII, VIII, IX and XII (Appendix E: Table E-1). The compounds formed during this phase, except for XI, appeared only as intermediates which underwent further transformation. Compound XI should be considered as the final MeCl-extractable product of the enzymatic transformation of SA, although traces of II were still present after 96 h of incubation (Figure 8-11i).

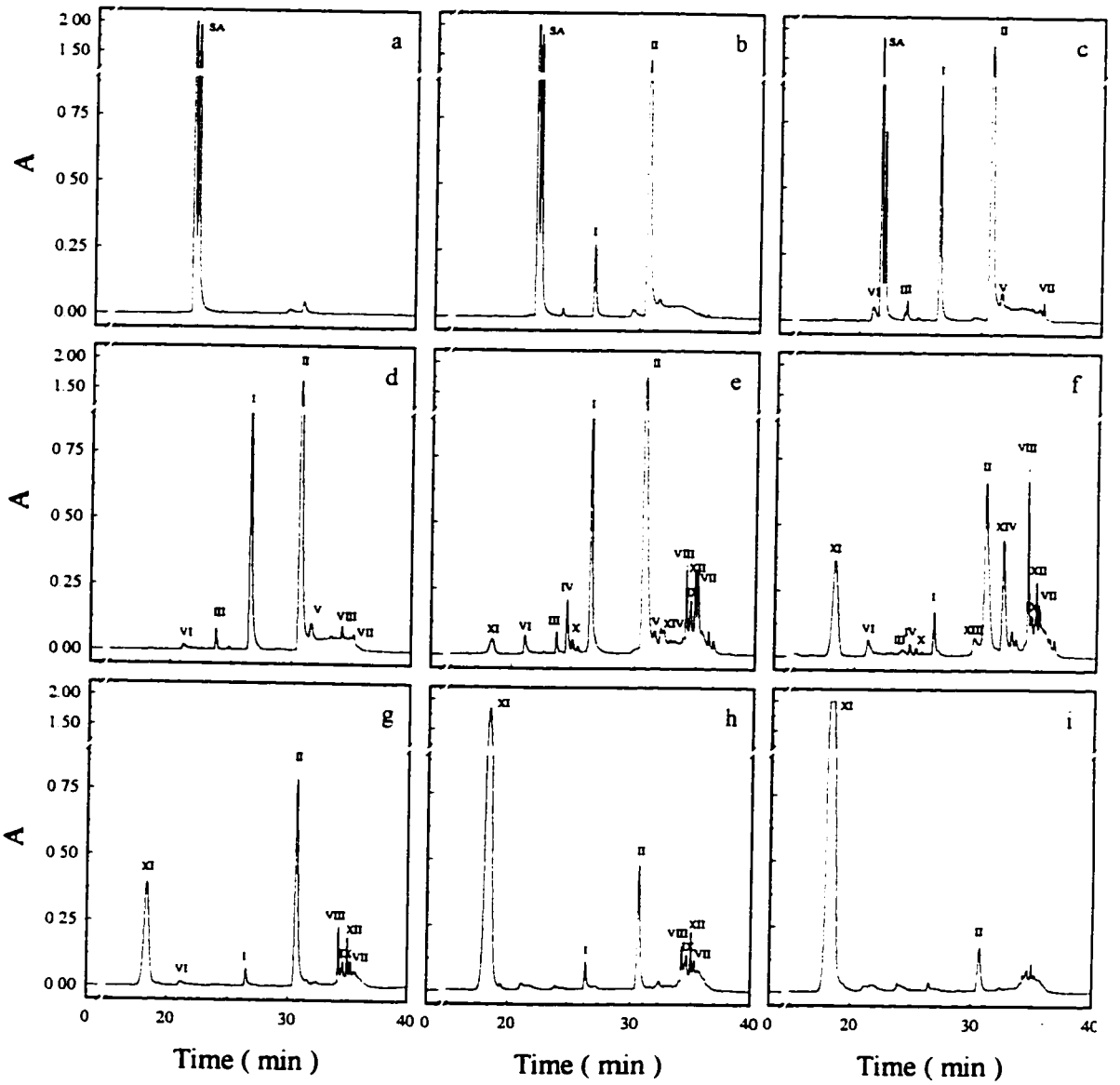


Figure 8-11. Formation of reaction products during the enzymatic transformation of sinapic acid. (a) initial; after: (b) 15 min.; (c) 20 min.; (d) 30 min.; (e) 1 h; (f) 3 h; (g) 8 h; (h) 20 h; (i) 96 h. Conditions described in *Experimental aspects*.

According to the results obtained, it was not certain whether all of the observed reactions had the enzymatic character. Some information regarding this aspect of the enzymatic transformation of SA was obtained from the product elucidation studies.

8.2.4 Product identification

To elucidate the structures of the products of the enzymatic transformation of SA, collected as fractions from HPLC separation, several analytical techniques were used, including ultraviolet-visible (UV-VIS), infra-red (IR) and nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and X-ray crystallography. The results obtained using these techniques will be described in this section.

8.2.4.1 Ultraviolet-visible spectroscopy

To obtain UV-VIS spectra of the detected products, the tunable absorbance detector used in the HPLC separation was replaced with the Photo Diode Array detector. The new detector simultaneously measured absorbencies of the sample at a defined range of wavelengths as the sample passed through the detector's optical cell. This allowed the UV-VIS spectrum of the effluent from the chromatographic column at the particular retention time to be obtained, thus providing the spectrophotometric data for the pure compounds represented by peaks on HPLC chromatogram. The obtained spectrophotometric data for the products I to XIV are presented in Appendix E (Table E-1).

8.2.4.2 Mass spectrometry

To obtain molecular masses of the collected products, the Mass Spectroscopy-Direct Probe (MS-DP) technique was used. The data that were obtained along with the sample spectra of compound II and XI are shown in Appendix E, Table E-4 and Figures E-1 and E-2, respectively. It should be mentioned that an attempt to determine the masses of all of the detected products was also undertaken using a gas chromatography-mass spectrometry (GC-MS) technique. Unfortunately, the GC-MS method had failed to produce any valuable results, mainly because the products could not be separated by the GC, even after their derivatization.

Note: The Liquid Chromatography-Mass Spectroscopy (LC-MS) method was also applied in this study to determine masses of all of the detected products. It produced very promising results, and in one instance it was used to confirm some of the results obtained with the DP technique. Unfortunately, the runs using LC-MS were performed in an outside laboratory using a different HPLC system, and there was not enough time to develop a specific chromatography method that would give a sufficient separation quality. Nevertheless, based on the data obtained, the LC-MS technique should be recommended for this kind of studies on this as well as on similar enzymatic systems.

8.2.4.3 Infrared spectroscopy

Since every compound having covalent bonds will absorb certain frequencies of electromagnetic radiation in the infrared region of the spectrum, the analysis based on this technique can provide data on the types of bonds present in the molecule.

The infrared analyses were performed in the National Research Council of Canada laboratories using the Fourier Transformed Infrared Spectroscopy technique. The obtained spectra for each of the products (fractions collected using HPLC) are presented in Appendix E (Figure E-3).

8.2.4.4 Nuclear Magnetic Resonance spectroscopy

Nuclear Magnetic Resonance spectrometry, NMR, is considered as the second, only to the X-ray crystallography, the most informative technique used in structure elucidation studies [Waterman and Mole, 1994]. Unfortunately one of the requirements for this technique is a relatively large sample size which in the case of carbon ^{13}C NMR has to be in the magnitude of several milligrams. Furthermore, the sample for the NMR study has to be as pure as possible, because NMR spectrum of a mixture is the sum of the spectra for all of the sample components. Thus, unless the composition of the analyzed sample is known, the interpretations of such a spectrum become fairly difficult. As it will be described later in the text, because of the complex composition of the reacting system it was only possible to obtain large quantities of compound XI and a mixture composed predominantly of compounds I and II. Since compound XI was identified based on the MS data (see later in the text) only the mixture of two I and II was subjected to the NMR analysis. Several batch transformations of SA were carried out to obtain enough material to run both proton ^{1}H and carbon ^{13}C NMR tests. The NMR analyses were performed at the Department of Chemistry, University of Ottawa. The obtained data are summarized in Appendix E (Table E-5).

8.2.4.5 X-ray crystallography

X-ray crystallography is considered the ultimate method for structure elucidation. Therefore, the final part in the structure elucidation studies involved X-ray crystallography analysis of compound II. The experimental details and the results obtained using this technique are given in Appendix E.

8.2.5 Structure elucidation

8.2.5.1 Characterization of products from Phase A of SA transformation

The identification of compounds I and II, the only major products formed during phase A of SA transformation, was based on the analysis of respective fractions collected from HPLC separations. The MS spectra for these two compounds showed that their molecular weights were 446 (Table E-4) which corresponds to the molecular formula of $C_{22}H_{22}O_{10}$. This indicated that these compounds could have been dimers of SA formed *via* subtraction of two hydrogen atoms from two SA molecules, and a subsequent coupling of the two phenoxy radicals formed. The formation of dimeric structures during transformations of syringic and vanilic acids with laccase was reported [Leonowicz *et al.*, 1984; Katase and Bollag, 1991]. The obtained MS fragmentation patterns indicated that compounds I and II were fairly symmetrical showing the most abundant mass fragment at m/z 181. This fragment was assigned to the 3,5-dimethoxy,4-hydroxy-α-ketone moiety [Kamaya *et al.*, 1983; Iwahara, 1983]. This result showed the existence of a phenoxy group, even though the formation of a carbonyl group was expected if I and II were dimers of SA formed by coupling of two phenoxy radicals. In addition, the appearance of fragments of 44, 402, and 358 in the mass spectra suggested the presence of one and two decarboxylated molecules, respectively [Pavia *et al.*, 1979]. This was, however, contradictory to the results obtained from FTIR analysis that did not confirm the presence (bands at 1680-1700 cm^{-1}) of a carboxylic group in either I or II. On the other hand, they showed a very well defined peak representing γ-lactone moiety, IR ν_{max}^{NaCl} cm^{-1} 1760 [Lacoste *et al.*, 1993], which could be responsible for the observed mass fragment of m/z 44. The absorption minimum at 3400 cm^{-1} along with the particular combination of neighboring peaks (Figure E-3) indicated that a phenoxy group with methoxy groups attached to the neighboring aromatic ring carbons was present in both I and II [Aldrich Atlas of Infrared

Spectra, 1989]. In general, the FTIR spectra of I and II, shown in Figure E-3 (Appendix E), are nearly the same and differed only by a presence of very small peaks at 1640cm^{-1}. The MS and UV-VIS data were also similar. Based on the MS and FTIR data, compound II was identified as r-1H2c,6c-bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3.3,0]-octane-4,8-dione, dehydrodisinapic acid dilactone, DAD. Transformations of SA to DAD have been already reported [Freudenberg and Shrade, 1955; Ahmed *et al.*, 1973; Rubino *et al.*, 1996], but these reactions were not enzymatic in nature, but were based on the oxidative coupling of SA in the presence of ferric chloride and oxygen. To confirm identity of II NMR analyses were performed. Since the fraction collection did not produce enough material for a NMR test, these tests were performed on the mixture of compounds I and II obtained after the extraction of the reacting solution of SA at the end of phase A. According to HPLC data, this mixture was predominantly composed of I and II, with II representing 90.0% of the sample. The chemical shifts and coupling constant obtained from these tests were assigned to the respective carbon and hydrogen atoms of compound II (Appendix E: Table E-5) based on a simulated spectra for DAD obtained using the ADC NMR software. The experimental chemical shifts obtained from the proton NMR are in good agreement with reported values [Ahmed *et al.*, 1973]. However, an X-Ray structure determination was made in order to rule out any error in the data interpretation. A crystal required for this test was grown in acetone from the sample used for NMR tests. The X-crystallography results confirmed that product II was dehydrodisinapic acid dilactone. Table E-6 (Appendix E) shows the relative position of atoms in DAD, as determined by X-ray crystallography, and the ORTEP is provided in Figure E-7 (Appendix E). These results showed that II was the stereoisomer of DAD with *cis* 'diequatorial' aryl substituents. This type of stereo-configuration was suggested by Ahmed *et al.* [1973] as the most thermodynamically stable form of DAD. The results obtained in this study confirm that hypothesis.

8.2.5.2 Characterization of products from Phase B of the transformation of SA

The products formed during phase B of the enzymatic transformation of SA were identified based on MS and FTIR data obtained using fractions collected during HPLC separation. The mass fragmentation patterns of compound VII (m/z 400), VIII (m/z 388), IX (m/z 388), XI (m/z 168),

XII (m/z 458) and XIV (m/z 400) are given in Table E-4 (Appendix E). Based on the MS data alone, only product XI was identified as 2,6-dimethoxy-*p*-benzoquinone by comparing the obtained results with the mass spectra of the authentic compound [Leonowicz *et al.*, 1984; Aldrich Library of Mass Spectra, 1980]. The remaining compounds did not have a match in the available databases and their structure elucidation was based entirely on the UV-VIS and IR spectroscopy, as well as the MS results. Considering compound VII, the spectrophotometric data (Appendix E: Table E-1) showed that it had a red colour, a characteristic property of some quinones. An FTIR spectrum showed that a 3,5-dimethoxy,4-hydroxy moiety and a γ-lactone group were present in the structure. However, the lactone peak was less intense as compared to that of DAD. The fragmentation in the mass spectrum of VII showed that the most abundant ion had a mass of 181 m/z, indicating the presence of a syringealdehyde-like moiety. According to these results, compound VII was identified either as 3-(3,5-dimethoxy-4-oxo-2,5-cyclohexadienylidenmethyl)-5-(4-hydroxy-3,5-dimethoxyphenyl)-2,5-dihydro-2-furanone, or as 5-(3,5-dimethoxy-4-oxo-2,5-cyclohexadienyliden)-3-(4-hydroxy-3,5-dimethoxyphenylmethyl)-2,5-dihydro-2-furanone (VIIa).

Concerning compound IX, the FTIR spectrum did not display a lactone group, but showed a very strong absorption band representing the aliphatic chain (peak at 2950 cm^{-1}). The spectrophotometric data (Appendix E: Table E-1) indicated a presence of carbonyl group (the red colour), but the respective band on FTIR spectrum was relatively weak. The fragmentation pattern in the mass spectrum (Appendix E: Table E-4) showed a hydroxy group (loss of the ion with mass 17 m/z). Based on these data, compound IX was identified as 4-(4-(3,5-dimethoxy-4-oxo-2,5-cyclohexadienyliden)-1,4-dihydroxy-(*E*)-2-butenylidene)-2,6-dimethoxy-2,5-cyclohexadien-1-one.

Although compounds VIII and XIV were probably isomers with compounds IX and VII, respectively, the fragmentation patterns and IR spectra (Appendix E: Figure E-3) showed large differences in their respective structures. Since neither the mass nor IR spectra of compounds VIII and XIV had a match in the available data bases these compounds remain unknown. Similarly, compound XII was also not identified.

8.2.6 Non-enzymatic and enzymatic transformations of DAD

The fact that Phase B of SA transformation starts when SA disappears from the system would suggest that this phase also has enzymatic character. Consequently, it can be expected that either SA acts as an inhibitor to the enzyme's active sites responsible for the transformations of I and DAD, or the affinity of the enzyme for these two isomers was much lower than for SA. To confirm the hypothesis that Phase B includes enzymatic reactions, compounds that would represent the reacting system at the end of Phase A, and which could be used as potential substrates for the enzyme, were required.

The extraction of the reacting solution at the end of phase A of the transformation of SA, resulted in a mixture composed predominantly of DAD and compound I - 90% and 9%, respectively, as determined by the HPLC. The mixture was used as a starting material to evaluate the nature of DAD transformation at the beginning of Phase B of the enzymatic oxidation of SA. The results obtained showed that DAD can be transformed either non-enzymatically or by the enzyme in the presence of oxygen.

Non-enzymatic transformation of DAD was caused by its thermolysis, and resulted in the formation of a new compound, XV. Thermolysis was noticeable only in the presence of water and did not take place when MeCl solutions of DAD were concentrated at temperatures up to 50°C. Since, compound XV showed a weak absorption maximum at 337 nm, this wavelength was used to investigate the extent of its thermolysis at different temperatures. The respective changes in the absorbance at 337 nm with time are presented in Figure 8-12. These data were described, assuming an equimolar reaction, by a first order reaction mechanism with the rate constant k_x. The values of k_x estimated by the non-linear least square method, were: 0.025, 0.054, 0.082, 0.143, and 1.111 min^{-1} at 20, 30, 40, 50, and 70°C, respectively.

The enzymatic character of the transformation of DAD is documented by changes in the UV-VIS spectra of a slightly hydrolyzed DAD (*i.e.*, mixture of DAD and XV) recorded after the addition of the enzyme (Figure 8-13). This enzymatic reaction resulted in the formation of the product/products having an absorbance maximum at 519 nm. The initial decrease in the absorbance at 337 nm indicated that XV was enzymatically transformed, which was confirmed by

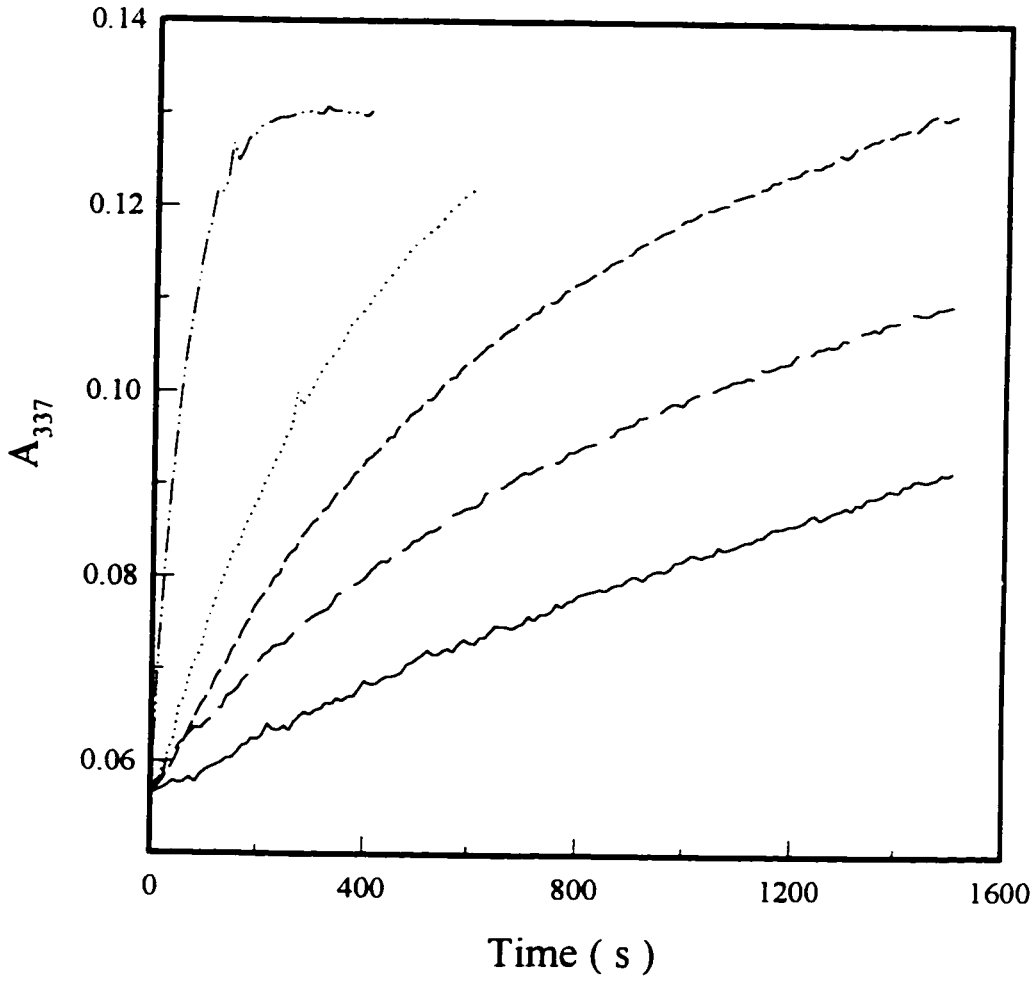


Figure 8-12. Changes in the absorbance at 337 nm caused by the thermolysis of DAD at: (——) 293 K; (— —) 303 K; (– – –) 313 K; (· · ·) 323 K; (– · ·–) 343 K.

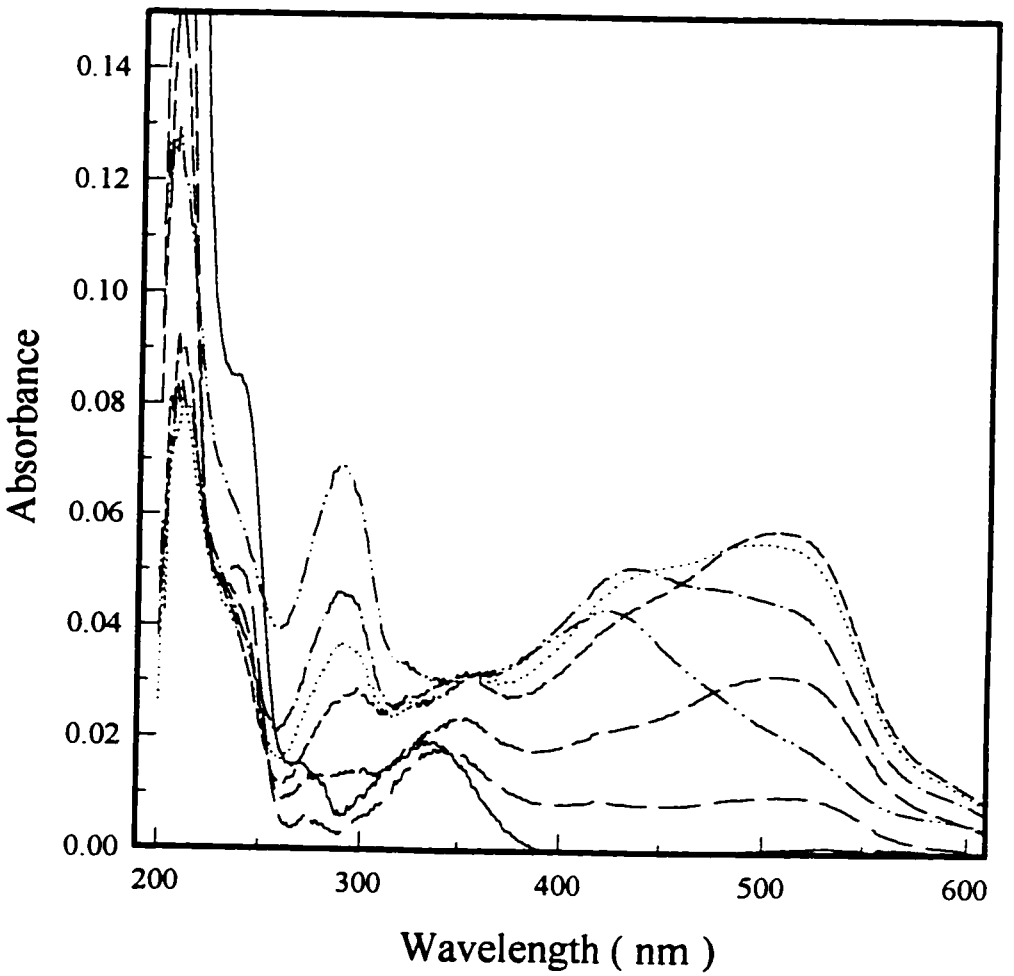


Figure 8-13. Changes in UV-VIS spectra of DAD after addition of enzyme from *Trametes versicolor*: (——) initial; (— —) 2 min.; (— —) 6 min.; (----) 20 min.; (·····) 1 h; (·– ·– ·) 7 h; (– · ·–) 12 h.

the disappearance of XV from the solution after the addition of the enzyme (data from HPLC analysis not shown here). It can be noted, that the structure of DAD is very similar to that of syringealdazine, a compound that is widely used to determine activities of laccases, and, therefore, DAD definitely represents a potential substrate for the enzyme used in this study. Furthermore, compound XV must have in its structure at least one hydroxy group attached to the aromatic ring to be enzymatically transformed [Khan and Overend, 1990].

To confirm the enzymatic character of the transformation of DAD, the changes in absorbance at 519 nm were used to evaluate the initial reaction rates at various substrate and enzyme concentrations, pH, and temperature. The results (Figure 8-14A-D) confirmed the enzymatic character of this reaction. For the enzyme concentration lower than 60.0% of the enzyme stock solution the initial reaction rate was directly proportional to its concentration, whereas for the higher enzyme concentrations there was a visible saturation pattern (Figure 8-14D). When the effect of substrate concentration was evaluated (Figure 8-14B) a pattern representing the initial part of a typical Michaelis-Menten type of kinetics was observed. The solubility limits for DAD did not allow for tests to be performed at higher substrate concentrations, and was also a reason for not evaluating of the enzyme kinetic constant. The optimum temperature and pH for this transformation were 45°C and 4.5, respectively (Figure 8-14 C and A). The intersection pattern observed in Figure 8-14C can be explained through the combined effect of temperature on the actual pH of the system, and the effect of pH on the enzyme stability.

8.2.7 Proposed reaction pathway

Considering all of the results obtained, the reaction pathway representing a part of the enzymatic transformation of SA can be proposed (Figure 8-15). The transformation starts through the formation of a phenoxy radical by the removal of hydrogen atom from the hydroxyl group of SA molecule. In the next step two radicals couple to give a product with a dimer-like structure. Since the radical formed can have five mesomeric forms 21 dimer-like products can be formed during a random coupling between two forms of the radical. The presence of DAD as a main product of the initial stages of SA transformation indicated, however, that a simple coupling of two radicals had to be followed by an intramolecular nucleophilic attack leading to the

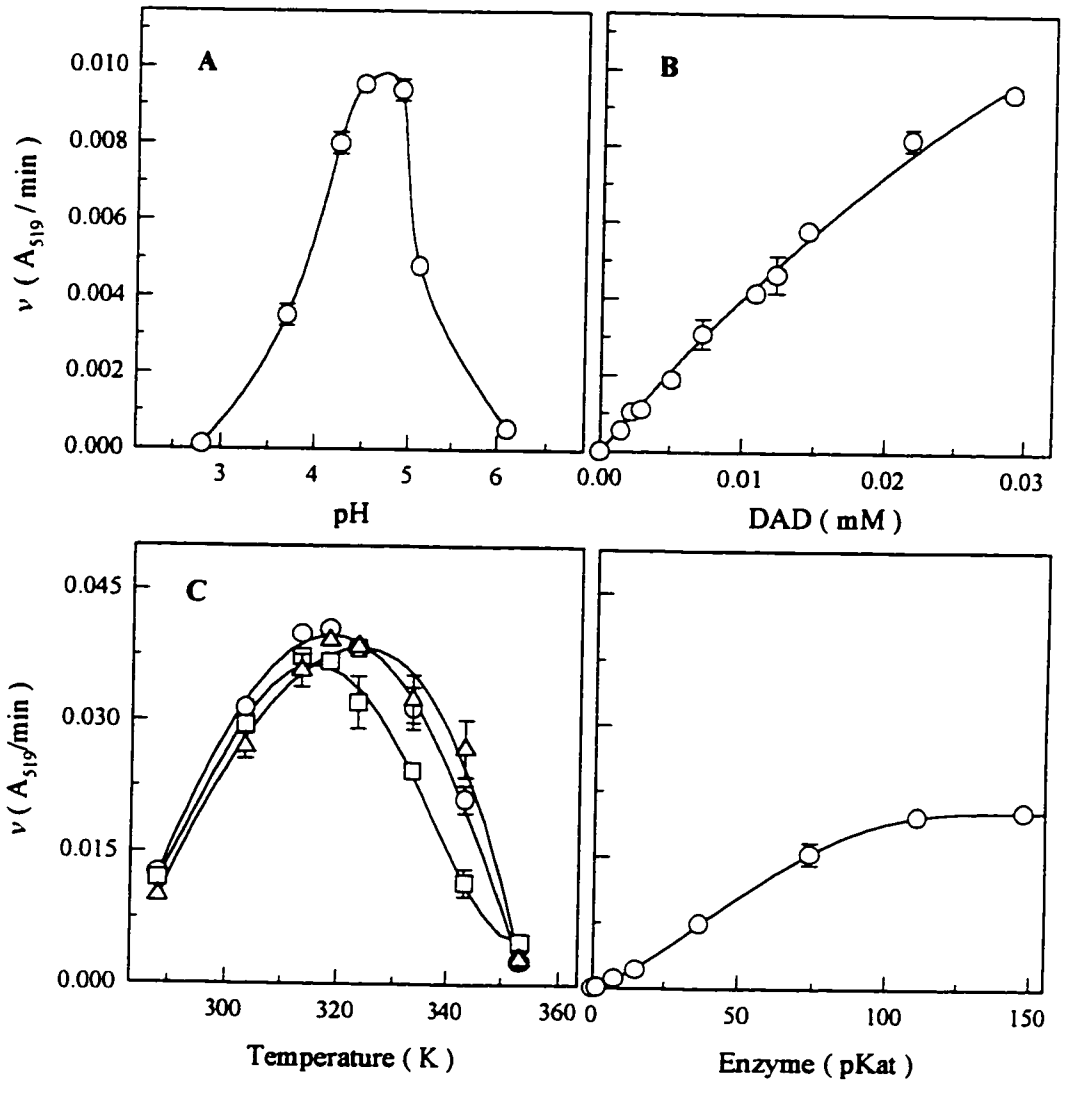


Figure 8-14. Effects of four system variables on the enzymatic transformation of DAD: pH (A); DAD concentration (B); temperature (C); enzyme concentration (D). Conditions: (A) T=30°C, DAD 0.028 mM, enzyme 14.7 pKat; (B) pH 4.5, T=30°C, enzyme 14.7 pKat; (C) O - pH 4.9, □ - pH 4.5, Δ - pH 4.25: DAD 0.014 mM, enzyme 36.9 pKat; (D) T=30°C, pH 4.5, DAD 0.01 mM.

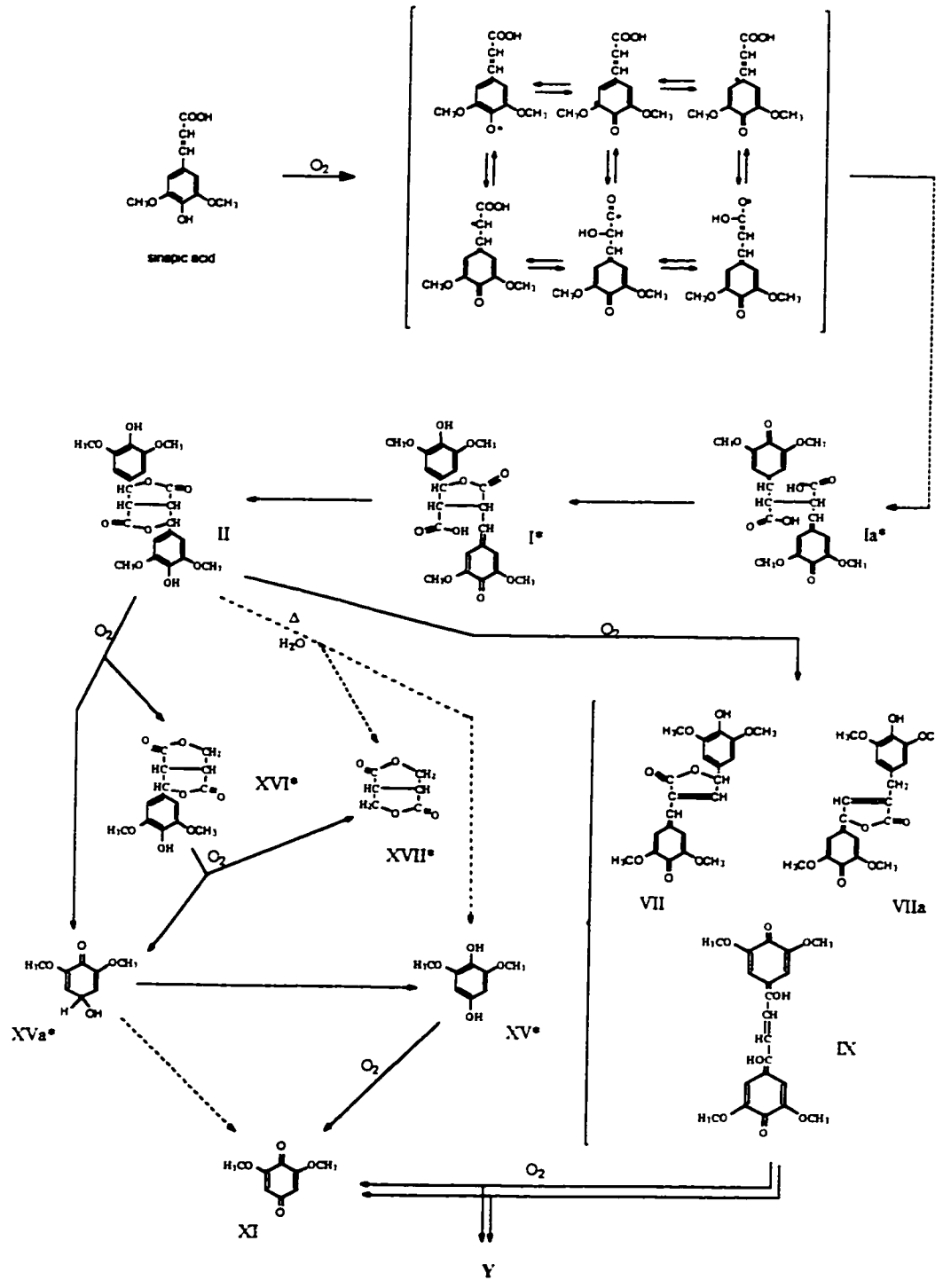


Figure 8-15. Proposed pathways for enzymatic transformation of sinapic acid by an enzyme preparation secreted by the white-rot fungus *T. versicolor*: ····· non-enzymatic reactions; —— enzymatic reactions. Symbol * next to the roman number indicates that such a compound represent only a suggested structure, and has no experimental verification. Letter Y represents unknown MeCl non-extractable products.

reappearance of phenoxy groups. It also showed that the radical coupling during the oxidation of SA took place in the β - β mode, rather than in the β - O-phenol positions as suggested by Choi and Sapers [1993]. The β - β mode of coupling was observed upon the formation of *d,l*-syringaresinol during the enzymatic transformation of sinapyl alcohol by a similar enzyme [Higuchi, 1991]. According to this kind of coupling mechanism, the first "dimer" formed should be a compound Ia, 2,3-bis-(3,5-dimethoxy-4-oxocyclohexadienylidenmethyl) succinic acid (Figure 8-15), which upon nucleophilic attacks rearranges to DAD. Considering that the second product, compound I, formed during phase A of the transformation of SA was the isomer of DAD with similar light absorbing properties and mass spectrum (Appendix E: Table E-1 and Table E-4, respectively), it can be speculated that compound I was an intermediate between Ia and DAD formed after only partial rearrangement of Ia (Figure 8-15). Compound I could also be a stereoisomer of DAD, however, a relatively big difference in the HPLC retention times between these two compounds (Appendix E: Table E-1) suggested the presence of more significant differences in their structures.

Phase B of the transformation of SA began with the enzymatic oxidation of DAD (Figure 8-13 and Figure 8-14) most likely by the same mechanism as the one responsible for the enzymatic oxidation of SA.

Bearing in mind that only a few of the separated products were identified, a part of the pathway (Figure 8-15) representing Phase B is highly theoretical. The structures of products from the enzymatic transformation of DAD, *i.e.*, compounds VII (m/z 400) and IX (m/z 388) should be confirmed, especially since the mechanism of the formation of IX is not clear. The final MeCl-extractable product of the overall transformation of SA is 2,6-dimethoxy-*p*-benzoquinone, product XI (Figure 8-11; Appendix E: Table E-4). Its formation from compounds IX and VII probably has an enzymatic character, even though the relatively low rate of this reaction (Figure 8-11) compared with the rate of SA transformation suggests otherwise. XI can also be formed through a pathway starting with the nonenzymatic transformation (thermolysis) of DAD (Figure 8-15). The identification of compound XV, a product of the thermolysis of DAD, was not performed in this work, however, since compound XV was enzymatically transformed it must have had in its structure at least one hydroxy group attached to the aromatic ring, a requirement for the substrates

for the enzyme used in this study [Khan and Overend, 1990]. In Figure 8-15 it is proposed that the thermolysis of DAD resulted in the elimination of the aryl groups from DAD and caused the formation of 2,6-dimethoxy-*p*-hydroquinone and compound XVII, 3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione. The formation of XVII during the transformation of *d,l*-syringaresinol by laccase was also suggested [Iwahara, 1983]. The other product of thermolysis, *p*-hydroquinone, which can be enzymatically oxidized to compound XI, can also be formed starting with the partial enzymatic oxidation of DAD (Figure 8-15). This step is followed by the coupling of two radicals, one hydroxy and one formed at the first carbon of the aromatic ring. Such a coupling would result in the cleavage of the aryl-alkyl bond and the formation of XVa, 4-hydroxy-2,6-dimethoxy-2,5-cyclohexadien-1-one that can further reacts to form XI and/or upon intramolecular rearrangement to form 2,6-dimethoxyphenol, and XVI, 3-(4-hydroxy-3,5-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione. Cleavage of the alkyl-aryl bond by the action of fungal oxidizing enzymes was reported [Kamaya *et al.*, 1981]. Compound XVI can also be produced during the thermolysis of DAD, however, it is rather questionable that an increase in temperature will cause the cleavage of the aryl-alkyl bond at one aromatic ring of DAD but not at the other.

Since sinapic acid, and its derivative sinapyl alcohol are precursors of the syringyl type of lignin [Kirk and Farrel, 1987], the presence of syringealdehyde as one of the metabolites was also expected. This compound is often reported as an intermediate during the degradation of lignin by phenol oxidizing enzymes. It could also be formed during thermolysis of DAD. However, a direct comparison of HPLC separation and spectrophotometric data of the authentic syringealdehyde with respective data for detected products did not confirm its presence.

The results obtained showed that the transformation of SA using the enzyme preparation secreted by *T. versicolor* takes place in at least two distinct phases. Each phase is initiated by a separate enzymatic step. In the first phase, SA is enzymatically transformed to two isomers of dehydrodisinapic acid dilactone. In this step one oxygen molecule is required to oxidized four SA molecules. The second product of this reaction is water. The second phase starts with the enzymatic transformation of DAD, most likely by the same mechanism as the one responsible for SA oxidation. Thermolysis of DAD complicates the overall reaction, since the product/products of this reaction is/are also enzymatically transformed. Assuming that all of the enzymatic reactions

take place at the same active site implies that during Phase A of SA transformation there are at least three possible substrates for the enzyme. Consequently, if these reactions can take place simultaneously, the free radicals formed as their products can randomly couple to produce a number of new compounds. These compounds can account for the products that are not extractable with MeCl, and which were not analyzed in this study. These organic-insoluble products are represented in Figure 8-15 by letter Y. Since their yield is 3 times larger than that of *p*-quinone it would be of great importance to perform extensive analytical work on this fraction.

8.2.8 Products of sinapine transformation

To determine whether the products formed during enzymatic transformation of SA are also formed when SIN is converted, the composition of a reacting solution of SIN and enzyme was monitored using HPLC. The results obtained showed (Figure 8-16) that only 2,6-dimethoxy-*p*-benzoquinone can be found in both systems.

As shown in Figure 8-16, the reaction pathway for SIN transformation is more complex than the one describing the conversion of SA. In the initial stages of the enzymatic conversion of SIN, nine products, compounds S1-S9, were detected. The dynamics of their formation is shown in Figure 8-17. According to these results compounds S5, S6, S7 and S8 should be considered as intermediate products that could be converted to *p*-quinone, whereas the compounds S1, S2, S3, S4 and S9 appeared to be final products which are formed from the beginning of SIN transformation. The results discussed here were obtained in the experiment where the initial SIN concentration was relatively high. This allowed higher concentrations of the products to be obtained, thus enhancing the possibility of their detection. However, these higher concentrations seemed to cause an inhibition of SIN transformation. This was confirmed by the results obtained when the reaction was carried out at higher temperature and 10 times lower initial SIN concentration. Under these conditions the total conversion of SIN was observed after 10 min of reaction, and the only product formed was 2,6-dimethoxy-*p*-benzoquinone. This result indicated that the reaction pathway of SIN transformation can be temperature depended. This phenomenon should be addressed in the future studies. For the time being, except for *p*-quinone, no other products of SIN transformation were identified.

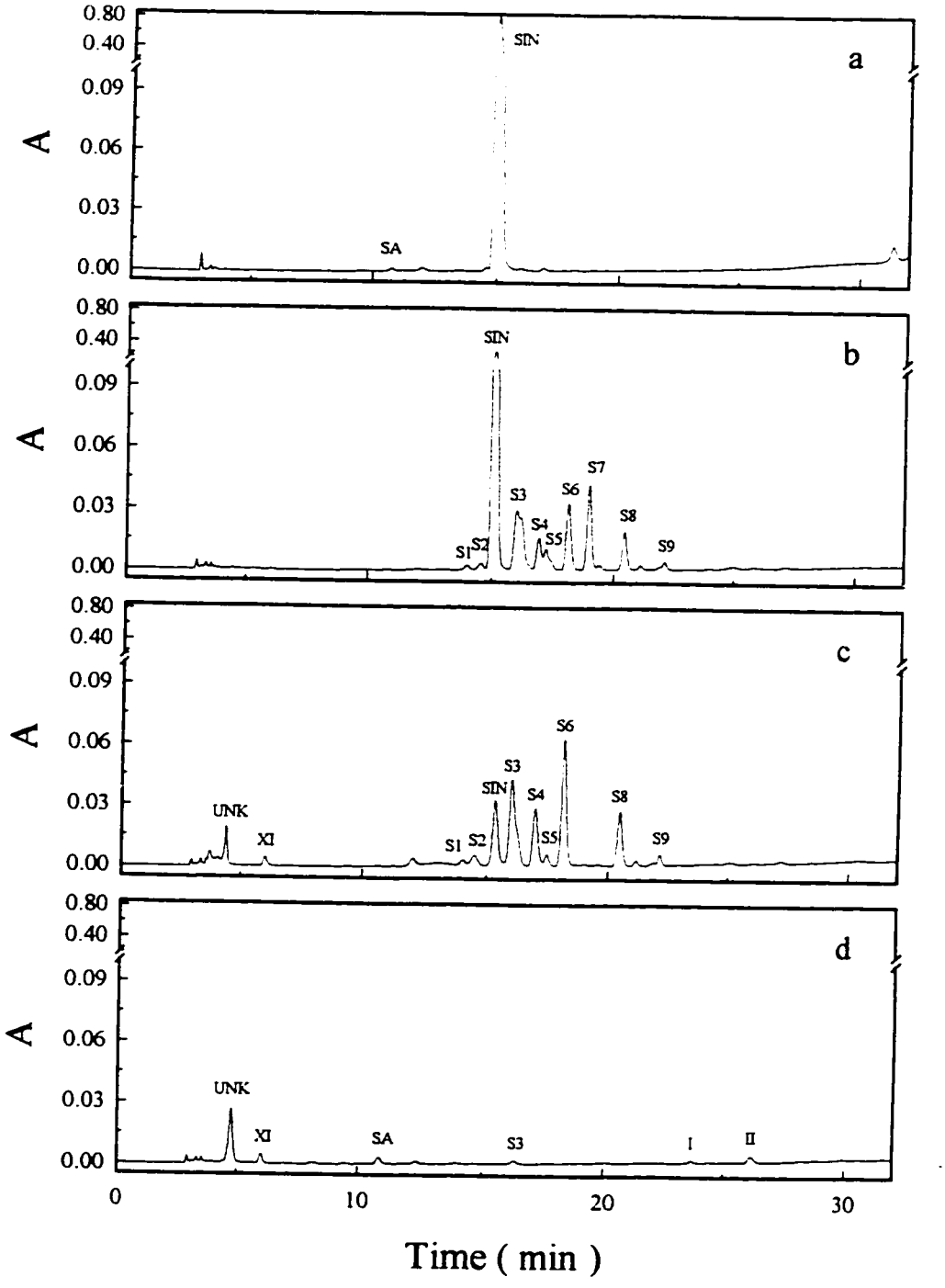


Figure 8-16. Comparison of the products of the enzymatic transformation of SIN: (a) initial; (b) 1 h; (c) 12 h; and SA: (d) 1 h. Both reactions were carried out at room temperature using 10 μL of enzyme stock solution per 1 mL of system. Initial concentration of: SIN 2.0 mM; SA 0.4 mM.

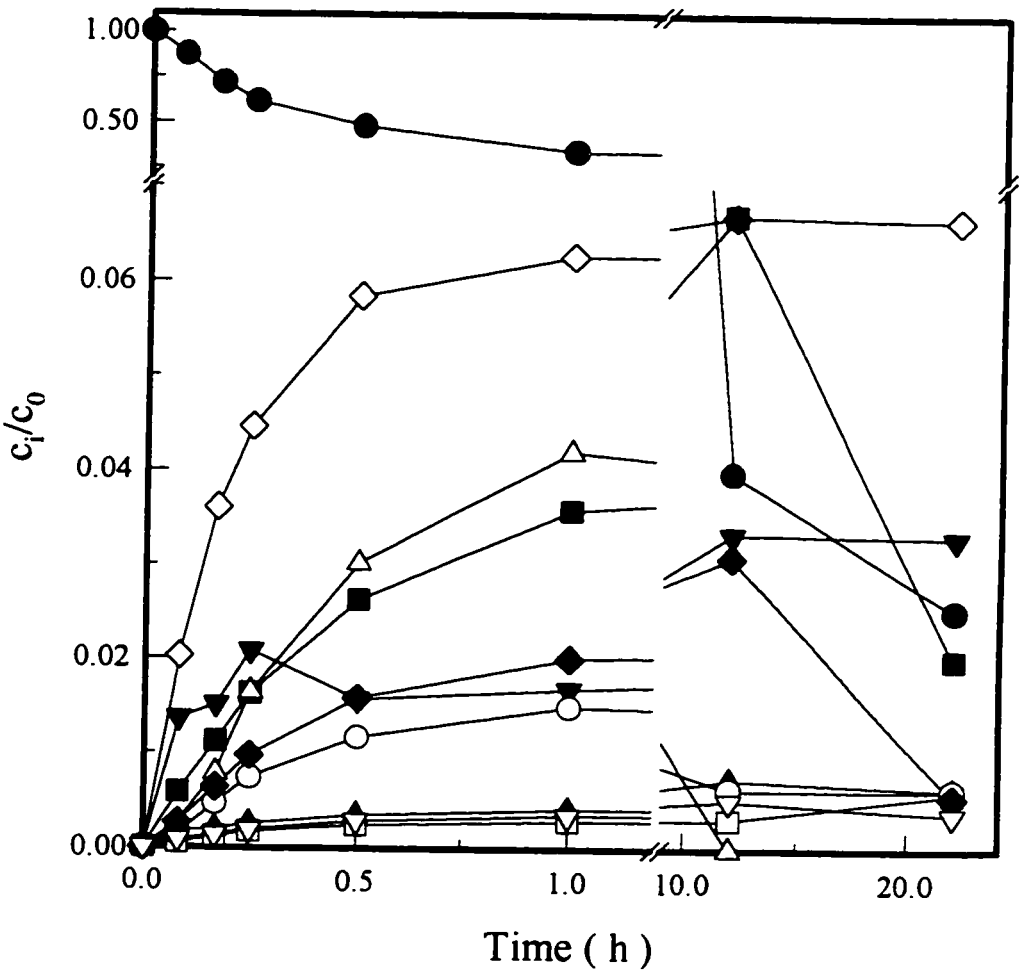


Figure 8-17. Dynamics of the formation of the products during SIN transformation: (●) SIN; (□) S1; (▲) S2; (◊) S3; (▼) S4; (○) S5; (■) S6; (Δ) S7; (◆) S8; (▽) S9. Conditions as for Figure 8-16.

8.3 Reaction mechanism for sinapic acid transformation

To describe mathematically the enzymatic transformations of SA and its derivatives, it was necessary to know the mechanisms of these reactions. Generally, oxido-reducing enzymes, such as polyphenol oxidase, transform phenolic substrates by reduction of molecular oxygen and subsequent oxidation of the substrates' phenoxy group. Considering that oxygen is utilized during these enzymatic reactions, the time changes in its concentration are often used to measure enzyme activity [Morohoshi and Haraguchi, 1987; Petersen and Degn, 1978; Rogalski *et al.*, 1990]. This method is suitable to evaluate the effects of most system variables on the enzyme activity, including phenolic and enzyme concentrations, pH, activators, etc. However, experimental results of the effect of temperature on enzyme activity can lead to wrong conclusions because the oxygen concentration strongly depends on temperature [Łącki and Duvnjak, 1996a]. Therefore, unless the enzyme is saturated with oxygen, the reaction rates measured at higher temperatures include effects from the decreased oxygen concentrations. In order to evaluate these effects, information regarding enzyme saturation with oxygen is required.

To determine the substrate-enzyme saturation condition for a two-substrate enzymatic system, a series of experiments with varying concentrations of one substrate at constant concentration of the other are required [Segel, 1975]. Although it is relatively easy to vary the phenolic concentration, varying the oxygen concentration requires special equipment. Petersen and Degn [1978] used an apparatus with a controlled composition of gas phase over the reaction mixture to evaluate the reaction of phenolic substrates with *Rhus* laccase. An experimental constraint was that their measurements had to be performed at relatively low oxygen concentrations to assure the enzyme was saturated with phenolic substrate. An alternative approach is to use a system with no gas head space which allows a desired initial oxygen concentration to be obtained regardless of the system temperature, hence, allowing the enzymatic rates to be evaluated at various concentrations of both substrates, regardless of their relative magnitude [Łącki and Duvnjak, 1996c].

As discussed in previous sections, the enzymatic transformation involved the utilization of oxygen and, therefore, to determine its mechanism, the studies with different oxygen concentrations

were required. For this purpose a special experimental setup, called a "closed" system, was designed and built. Its detailed description is given in *Chapter 3*. Using this experimental apparatus the mechanism by which sinapic acid is transformed by polyphenol oxidase secreted by *T. versicolor* was studied. In addition, the effect of temperature on the enzyme activity at constant initial oxygen concentration was evaluated, and the obtained results were compared with those obtained by spectrophotometric techniques (section 8.1.5) in which the initial oxygen concentration varied with temperature.

8.3.1 Determination of reaction mechanism for SA transformation

Preliminary modeling trials showed that it was possible to describe the experimental data by several mechanisms for a bireactant system. However, some of the values obtained for the kinetic parameters did not seem to be physically meaningful. Therefore, in order to narrow the number of reaction mechanisms, the experiment based on the measurements of the initial rates was designed. The mechanism elucidated by this method was then used to describe the effect of temperature on the SA transformation by applying transient techniques. The initial rate measurement method involved the evaluation of the effects of initial SA and oxygen concentrations on the rate of oxygen disappearance in the "closed" system by varying one substrate concentration at fixed levels of the other. In total, 25 runs conditions were tested. In these runs, only changes in the oxygen concentration were monitored. The initial reaction rates, calculated from the linear part of the oxygen progress curves, are shown in Figure 8-18 A and B as functions of SA and oxygen concentrations, respectively. The results indicate that the enzyme can be apparently saturated with one substrate if the concentration of the other is low.

A lower concentration of SA, compared to oxygen, is required to saturate the enzyme. The data also show that in the case of an "open" system, such as a spectrophotometric cuvete, simultaneous saturation of the enzyme with both substrates cannot be attained because of oxygen solubility limits.

The results shown in Figure 8-18 A and B are also presented as Hanes-Wolff plots (Figure 8-18 C and D) which show intersection patterns that are characteristic for at least three reaction mechanisms [Segel, 1975]. Two of them, Ordered Bi-Bi and Theorell-Chance Bi-Bi mechanisms,

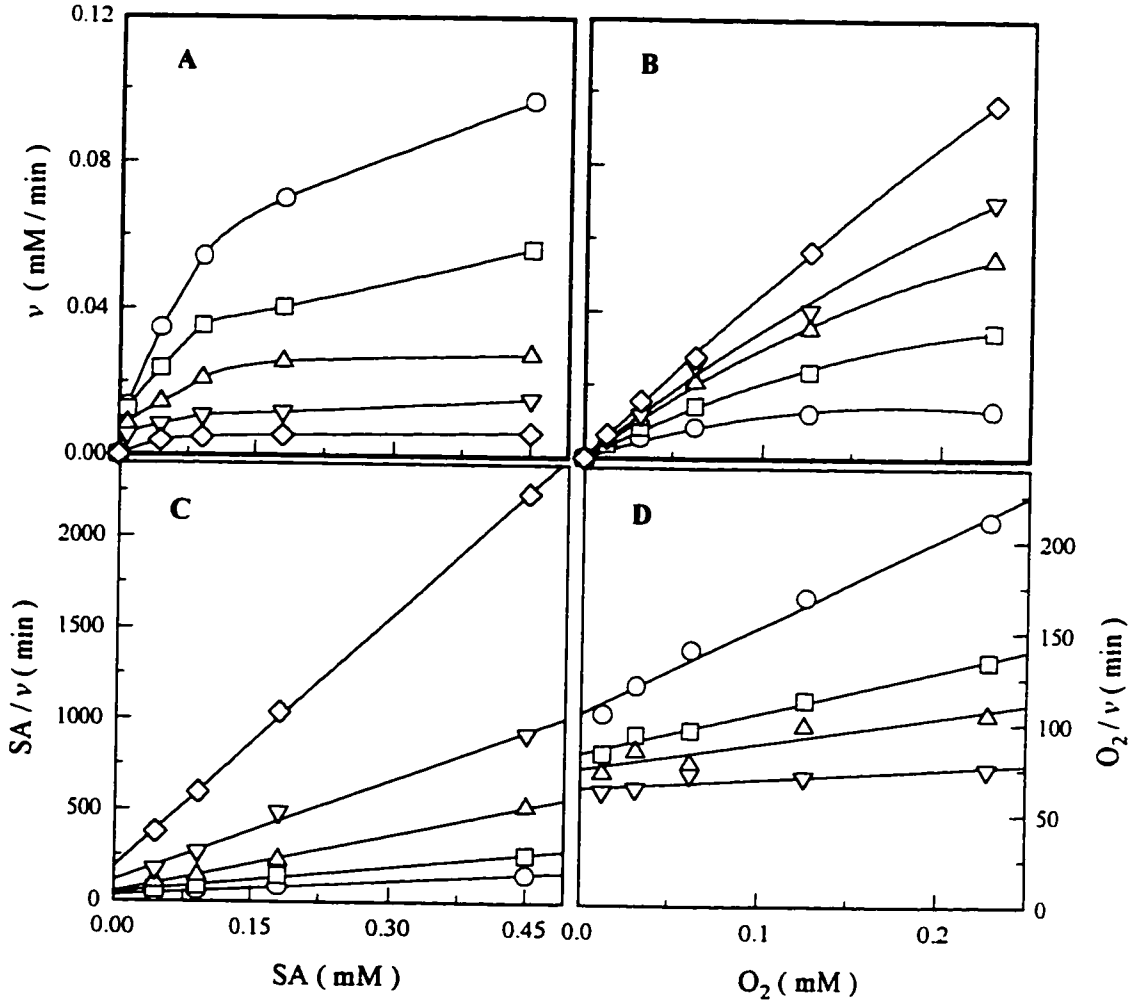


Figure 8-18. Effects of the oxygen (A) and sinapic acid (B) concentrations on the initial reaction rate at 30°C. The respective Hanes plots (C) and (D) of the data shown in Figures A and B. Oxygen concentrations Figures A and C: (O) 0.2280 mM; (□) 0.1250 mM; (Δ) 0.0625 mM; (▽) 0.0312 mM; (◊) 0.0125. Sinapic acid concentrations Figures B and D: (◊) 0.45 mM; (▽) 0.180 mM; (Δ) 0.090 mM; (□) 0.045 mM; (O) 0.009 mM.

are based on the quasi steady-state approximation, whereas the third, which is called a Random Bireactant Mechanism, is based on the rapid-equilibrium assumption. To distinguish among these mechanisms, studies of product inhibition patterns are required [Segel, 1975]. Unfortunately, such experiments are unlikely to be performed for polyphenol oxidase because the initial products of phenolic transformation are most likely highly reactive free radicals, that instantly polymerize, and water which is already in excess in the system [Łącki and Duvnjak, 1996c]. Bollag and coworkers [Bollag *et al.*, 1982; Katase and Bollag, 1991] showed that a laccase secreted by *T. versicolor* transformed vanillic and syringic acids by forming free phenoxy radicals as intermediates. The same reaction pathway was elucidated in this work (Figure 8-15).

The Random Bireactant Mechanism is not likely to occur, especially if it is assumed that the enzyme exists only in one reduced form that requires oxidation with oxygen; therefore, the other two mechanisms (Figure 8-19 A and B) have to be considered. In the absence of the products, P and Q, the velocity equation that describes these systems is the same and given by Eq.(8.2) [Segel, 1975]:

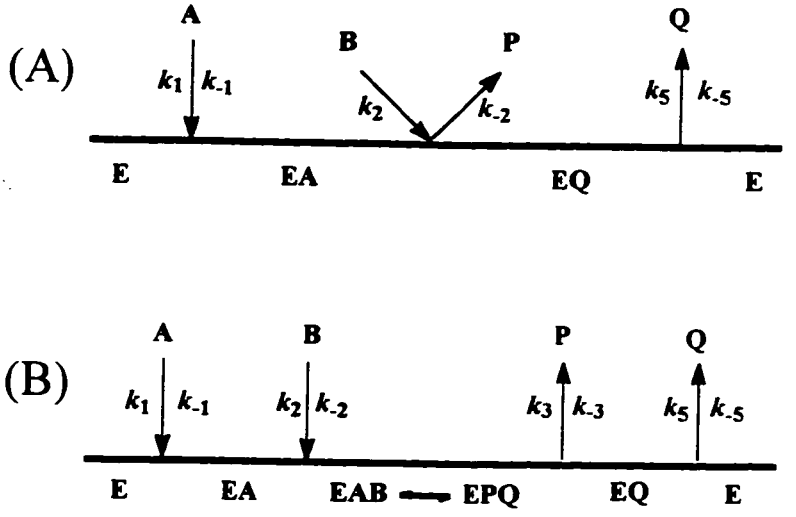


Figure 8-19. (A) - Theorell-Chance Bi-Bi mechanism; (B) - Ordered Bi-Bi mechanism.

$$v = \frac{V_{max}}{1 + \frac{K_{m,A}}{A} + \frac{K_{m,B}}{B}\left(\frac{K_{i,A}}{A} + 1\right)} \qquad (8.2)$$

The definitions of the respective constants in Eq.(8.2) for each of the two mechanisms are displayed in Table 8-3.

The reaction products are considered absent because: (1) if it is assumed that oxygen is substrate A (meaning that oxidation of the enzyme active site is necessary for the reaction to occur), then product P (Figure 8-19 A and B) is a free radical, the concentration of which can be considered zero because of its instantaneous polymerization; and (2) product Q is water, the effect of which can be neglected because it is already in excess.

Eq.(8.2) can be simplified to the typical Michaelis-Menten form when only one substrate is varied. In the case when A is kept constant, the initial velocity equation expressed through the apparent Michaelis-Menten constant is given by Eq.(8.3).

$$v = \frac{V_{max}^{app}}{1 + \frac{K_{m,B}^{app}}{B}} = \frac{\frac{V_{max}}{1 + \frac{K_{m,A}}{A}}}{1 + \frac{K_{m,B}}{B}\frac{A + K_{i,A}}{A + K_{m,A}}} \tag{8.3}$$

The integrated form of Eq.(8.3) would give the change in the concentration of the substrate B with time. However, when the concentration of A changes during the reaction, this enzymatic transformation has to be described by the system of ordinary differential equations that also accounts for these changes. Furthermore, if the enzyme deactivates significantly, the change in its concentrations with time must be included in the analysis. In the case when there are no additional

Table 8-3. Definitions of the respective constants for the two mechanisms described by Eq.(8.2).

Mechanism	V_{max}	$K_{m,B}$	$K_{m,A}$	$K_{i,A}$	$K_{m,XV}$	$K_{m,DAD}$
Ordered Bi-Bi	$\frac{k_3 k_5}{k_3 + k_5} E_t$	$\frac{k_5(k_{-2} + k_3)}{k_2(k_3 + k_5)}$	$\frac{k_3 k_5}{k_1(k_3 + k_5)}$	$\frac{k_{-1}}{k_1}$		
Theorell-Chance Bi-Bi	$k_5 E_t$	$\frac{k_5}{k_2}$	$\frac{k_5}{k_1}$	$\frac{k_{-1}}{k_1}$	$\frac{k_5}{k_4}$	$\frac{k_5}{k_3}$

where: $E_t = \beta \times v_{enz}/V_{sys}$. The definition for the constants $K_{m,XV}$ and $K_{m,DAD}$ as defined for the proposed reaction system given in Figure 8-20.

reactions involving either oxygen or SA, the rates of their disappearance during the enzymatic reaction would be described by Eq.(8.2) with a modification based on the reaction stoichiometry. However, during the course of this work, a steady decrease in the oxygen concentration was noticed even after SA disappearance. Considering that the decrease was much faster than would be caused by the Clark's electrode only, the observed changes in the oxygen concentration had to be associated with reactions involving oxygen and the products from the SA transformation.

8.3.2 Overall reaction mechanism - Phase A and Phase B

As discussed in section 8.2.7, two products of the enzymatic transformation of SA that are enzymatically transformed in the presence of oxygen are dehydroxydisinapic acid dilactone, DAD, and 2,6-dimethoxy-*p*-hydroquinone, XV. Assuming that the enzymatic transformations of DAD and XV proceeded according to the same reaction mechanism as the one for SA, then Phase A and Phase B of the enzymatic transformation of SA can be represented by the Theorell-Chance Bi-Bi mechanism with three alternate oxidizable substrates (Figure 8-20) [Łącki and Duvnjak, 1996c]. This mechanism was chosen because of its simpler form with respect to the number of the respective rate constants (Table 8-3) and their estimation.

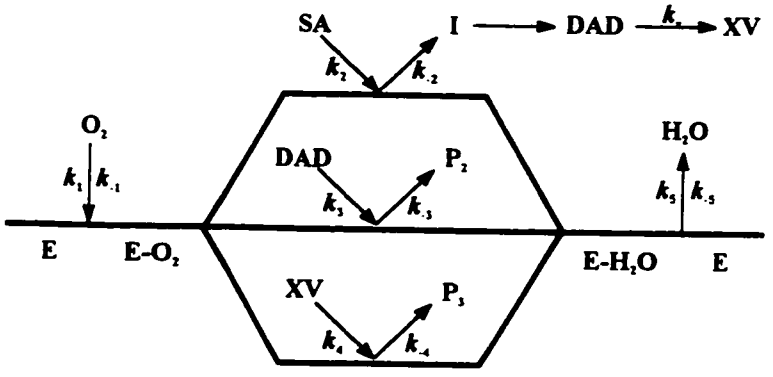


Figure 8-20. Proposed reaction pathway for the transformation of sinapic acid based on Theorell-Chance Bi-Bi mechanism with three alternate substrates for the oxygen-enzyme complex, $E\text{-}O_2$.

The enzymatic system presented in Figure 8-20 is described by the system of ordinary differential equations (ODE) given by Eq.(8.4)-Eq.(8.8):

$$-\frac{dV_{max}}{dt} = k_d V_{max} \tag{8.4}$$

$$-\frac{d[SA]}{dt} = \frac{mV_{max}}{1+\frac{K_{m,O_2}}{[O_2]}+\frac{K_{m,SA}}{[SA]}\left(\frac{K_{i,O_2}}{[O_2]}+1\right)+\left(\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}+\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}\right)\left(1+\frac{K_{m,O_2}}{[O_2]}\right)} \tag{8.5}$$

$$-\frac{d[O_2]}{dt} = -k_P[O_2]+\gamma k_L a\left(C^{*}_{O_2}-[O_2]\right)$$

$$+\frac{V_{max}\left(1+\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}+\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}\right)}{1+\frac{K_{m,O_2}}{[O_2]}+\frac{K_{m,SA}}{[SA]}\left(\frac{K_{i,O_2}}{[O_2]}+1\right)+\left(\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}+\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}\right)\left(1+\frac{K_{m,O_2}}{[O_2]}\right)} \tag{8.6}$$

$$\frac{d[DAD]}{dt} = -k_x[DAD]$$

$$+\frac{mV_{max}\left(1-\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}\right)}{1+\frac{K_{m,O_2}}{[O_2]}+\frac{K_{m,SA}}{[SA]}\left(\frac{K_{i,O_2}}{[O_2]}+1\right)+\left(\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}+\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}\right)\left(1+\frac{K_{m,O_2}}{[O_2]}\right)} \tag{8.7}$$

$$\frac{d[XV]}{dt} = k_x[DAD]$$

$$-\frac{mV_{max}\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}}{1+\frac{K_{m,O_2}}{[O_2]}+\frac{K_{m,SA}}{[SA]}\left(\frac{K_{i,O_2}}{[O_2]}+1\right)+\left(\frac{K_{m,SA}}{K_{m,DAD}}\frac{[DAD]}{[SA]}+\frac{K_{m,SA}}{K_{m,XV}}\frac{[XV]}{[SA]}\right)\left(1+\frac{K_{m,O_2}}{[O_2]}\right)} \tag{8.8}$$

The factor m (Eq.(8.5), Eq.(8.7) and Eq.(8.8) represents the number of molecules of SA, DAD, or XV that have to be oxidized in order to reduce one molecule of oxygen to water. It is necessary to note that the assumption regarding the stoichiometry of the reactions involving DAD and XV may not be correct, but until the exact mechanism for these transformations is known, it

can be taken as a reasonable approach. The second term in Eq.(8.6) describes the changes in the oxygen concentration due to the first-order reaction when measuring oxygen in the Clark's electrode [Holtzapple *et al.*, 1989], whereas the third term accounts for the diffusion of oxygen into the reaction system. The parameter γ in this term characterizes the closed and open reaction systems and can have only two values, 0 or 1, respectively. Thermal deactivation of the enzyme is introduced through Eq.(8.4). First-order, irreversible deactivation was used because it accurately described the results of the thermal deactivation of this enzyme at pH 3.5 (Figure 8-21). Because the actual molar enzyme concentration in the stock solution is unknown, a parameter β [millimoles of enzyme per mL of the enzyme stock solution] (Table 8-3) was introduced. For the purpose of parameter estimation, β was set to be equal 1.25×10^{-4} M. This value has no experimental basis, but seems to be within reasonable range of concentrations. For instance, Khan and Overend [1990] reported a 20-fold increase in the enzyme activity upon partial purification. The system of ODE's is reduced to four equations because Eq.(8.4) can be solved independently, with the usual initial conditions, and the result substituted back into the other equations.

A better picture of this reacting system would have been obtained if the concentrations of all of the compounds included in the proposed reaction pathway had been known and used to estimate the respective rate constants. Unfortunately, the HPLC separation method, which gave very good results in terms of selectivity and separation when used to detect DAD and XV in the methylene chloride extracts from the reaction, did not give conclusive results for these compounds when the aqueous sample was taken from the "closed" system. This was caused by the limited solubility of compound DAD in water, which affected both its sampling and separation. Therefore, the results obtained for compounds DAD and XV were not used for the parameter estimation. They are, however, presented (in terms of the concentrations calculated from the area of the respective peaks using the SA standard curve) in all of the respective figures.

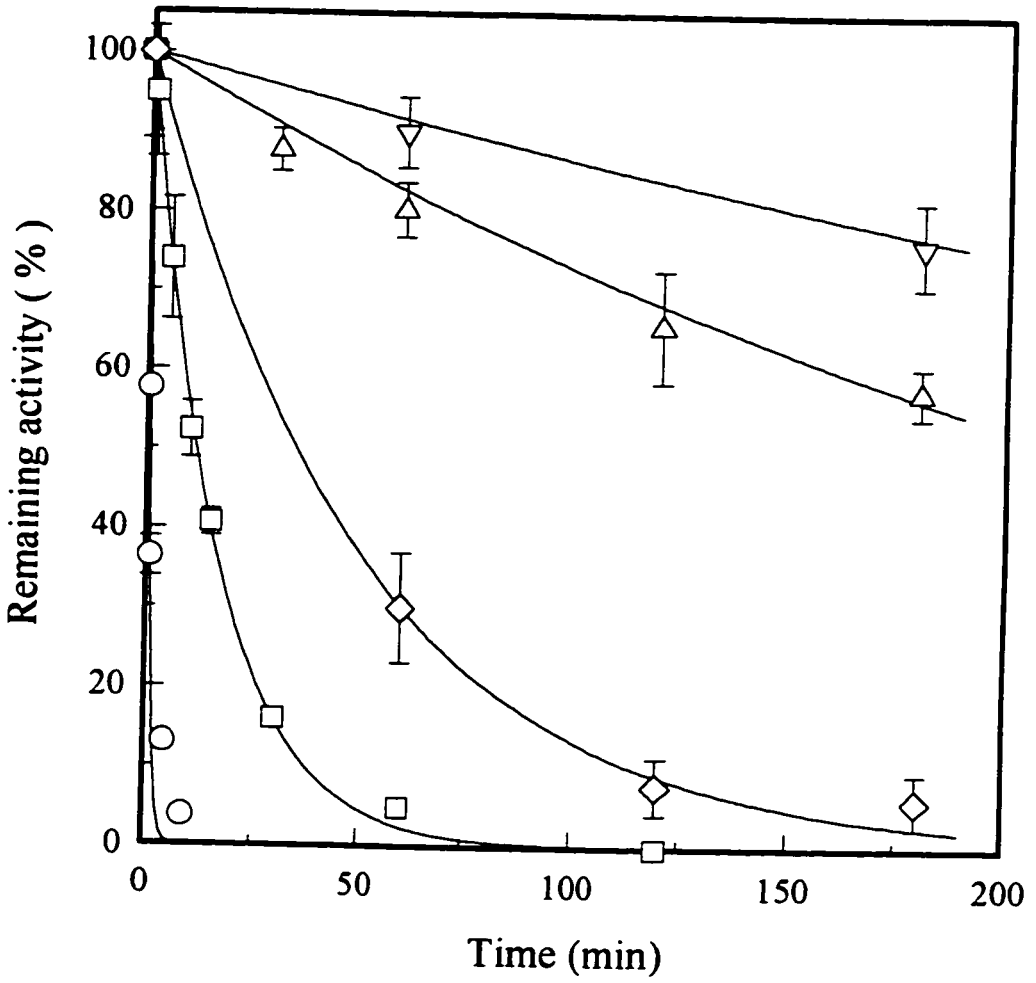


Figure 8-21. Effect of temperature on the stability of the enzyme preparation preincubated at pH 3.5 and at: (▽) 30°C, (△) 40°C, (◊) 50°C, (□) 65°C, (○) 80°C; lines are the predicted data using a first-order deactivation mechanism (Eq.(8.4)).

8.3.3 Parameter estimation

Only the rate constants describing the enzymatic reactions presented in Figure 8-20 were estimated by fitting Eq.(8.4)-Eq.(8.8) to the data collected in the "closed" system. The parameters k_d, k_x, k_p, and k_La, were independently estimated using data from separate experiments, namely preincubation (Figure 8-21), thermolysis (Figure 8-12), Clark electrode oxygen consumption, and the oxygen mass transfer coefficient evaluation (data for the last two experiments not shown). Furthermore, the preliminary estimation runs showed that the factor m varied between 3.92 and 4.05 for all of the experimental data used in the analysis. According to the reaction stoichiometry, this factor must be an integer and, therefore, it was set to 4. The rate constants at 33°C were obtained using all the data collected at this temperature in the "closed" system, and although these constants differ slightly from those obtained when each run was analyzed separately, they represent more accurate estimates. All of the parameter estimates obtained in this work are given in Table 8-4. They were used to calculate the predicted progress curves shown in the respective figures.

8.3.4 Closed system

Experiments in which the initial oxygen concentration was varied at a constant temperature and the temperature was varied at a constant oxygen concentration were performed to validate the proposed model. The results were used to obtain the least square estimates of the model parameters (Table 8-4).

Table 8-4. Estimated values for the kinetic constants in Eqs.(8.4)-(8.8) at three temperatures.[a]

T [K]	k_5 [min^{-1}]	K_{m,O_2} [mM]	$K_{m,S4}$ [mM]	K_{i,O_2} [mM]	$K_{m,DAD}$ [mM]	$K_{m,XV}$ [mM]	k_x [min^{-1}]	k_d [min^{-1}]	k_p [min^{-1}]	k_La [min^{-1}]
293.2	28.5	0.1426	0.0181	0.0099	1.4414	0.5983	0.0004	0.0004	0.0	-
306.2	130.1	0.6211	0.0195	0.0278	1.5013	0.2417	0.0017	0.0019	9.9×10^{-5}	0.0485
318.2	1860.4	8.4893	0.0671	0.0522	3.5695	0.2966	0.0034	0.0079	2.5×10^{-4}	-

[a] factor m = 4; v_{enz} = 0.1 mL; β = 1.25 x 10^{-4} mmol/mL.

8.3.4.1 Effect of initial oxygen concentration

The rate of SA transformation strongly depended on the initial oxygen concentration (Figure 8-22 A and B). Decreased oxygen concentrations significantly reduced the reaction rate, which is consistent with the results presented in Figure 8-18. Furthermore, Figure 8-22 B shows that the SA transformation was incomplete because of the lack of oxygen when the ratio of the initial concentration of SA to oxygen was larger than 4. This confirms that four molecules of SA are required to reduce one molecule of oxygen to water. This also shows that a proton is extracted from the phenoxy group of SA forming the free radical. As shown in Figure 8-22, the proposed model accurately described experimental data for oxygen and SA. The big discrepancy between the predicted and experimental results regarding compound DAD is most likely caused by the difficulties related to its solubility, as discussed earlier. Nevertheless, the experimental and predicted results for DAD and XV showed some similarities indicating that the proposed reaction pathway is possible, even though it may be oversimplified.

8.3.4.2 Effect of Temperature

Considering that the solubility of oxygen in an open system depends on the temperature, the effect of this variable on the reaction progress at a constant initial oxygen concentration was evaluated. The results obtained are displayed in Figure 8-23 A-C. These data were used to estimate model parameters at a given temperature (Table 8-4). Temperatures above 45°C were not investigated because of the practical limitation of the Clark's electrode. The model predictions agree well with the experimental results for oxygen and SA at all three temperatures. Furthermore, the predicted results for DAD and XV, qualitatively agree with the results obtained, showing an increase rate of formation for those compounds at higher temperatures. The discrepancy between the predicted and experimental results can be explained as was done for Figure 8-22 A and B.

Assuming that the reaction proceeds according to the mechanism presented in Figure 8-20, the results in Table 8-4 can be expressed in terms of the respective rate constants using the relationships from Table 8-3. These rate constants presented in the Arrhenius plot (Figure 8-24) show straight-line relationships. The respective activation energies, $E_{a,i}$, and frequency factors, $k_{0,i}$,

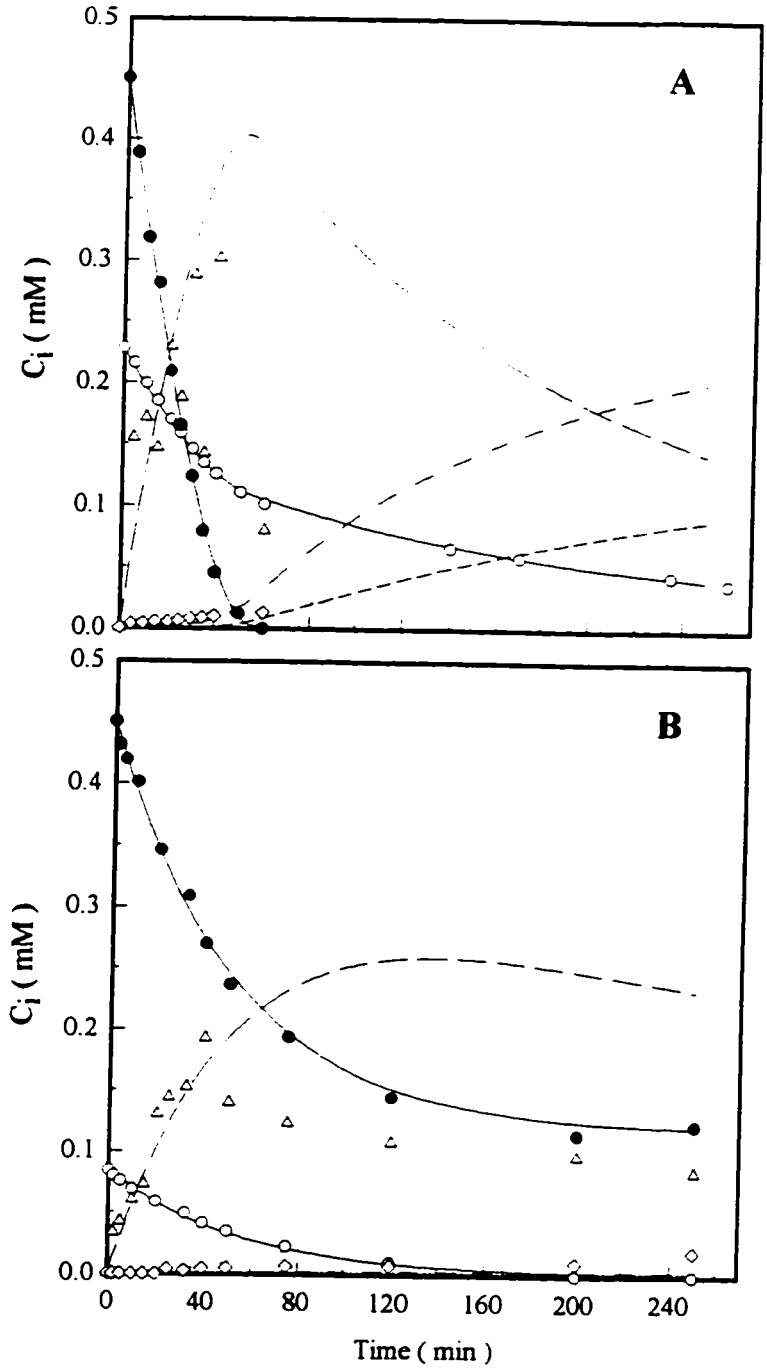


Figure 8-22. Effect of the initial oxygen concentration on the concentration-time curves for sinapic acid and oxygen. Oxygen concentrations: (A) 0.228 mM; (B) 0.084 mM. Symbols: (○) oxygen; (●) SA; (Δ) DAD; (◊) XV. The lines are the predicted data by Eqs.(8.4)-(8.8): (———) O_2 and SA; (·····) XV; (— — —) DAD; (·—·—·—·) P_2; (– – – –) P_3. Conditions: pH 3.5, 33°C, and e_0 = 0.1 mL. The measured concentration of compound DAD was multiplied by 5 for clarity.

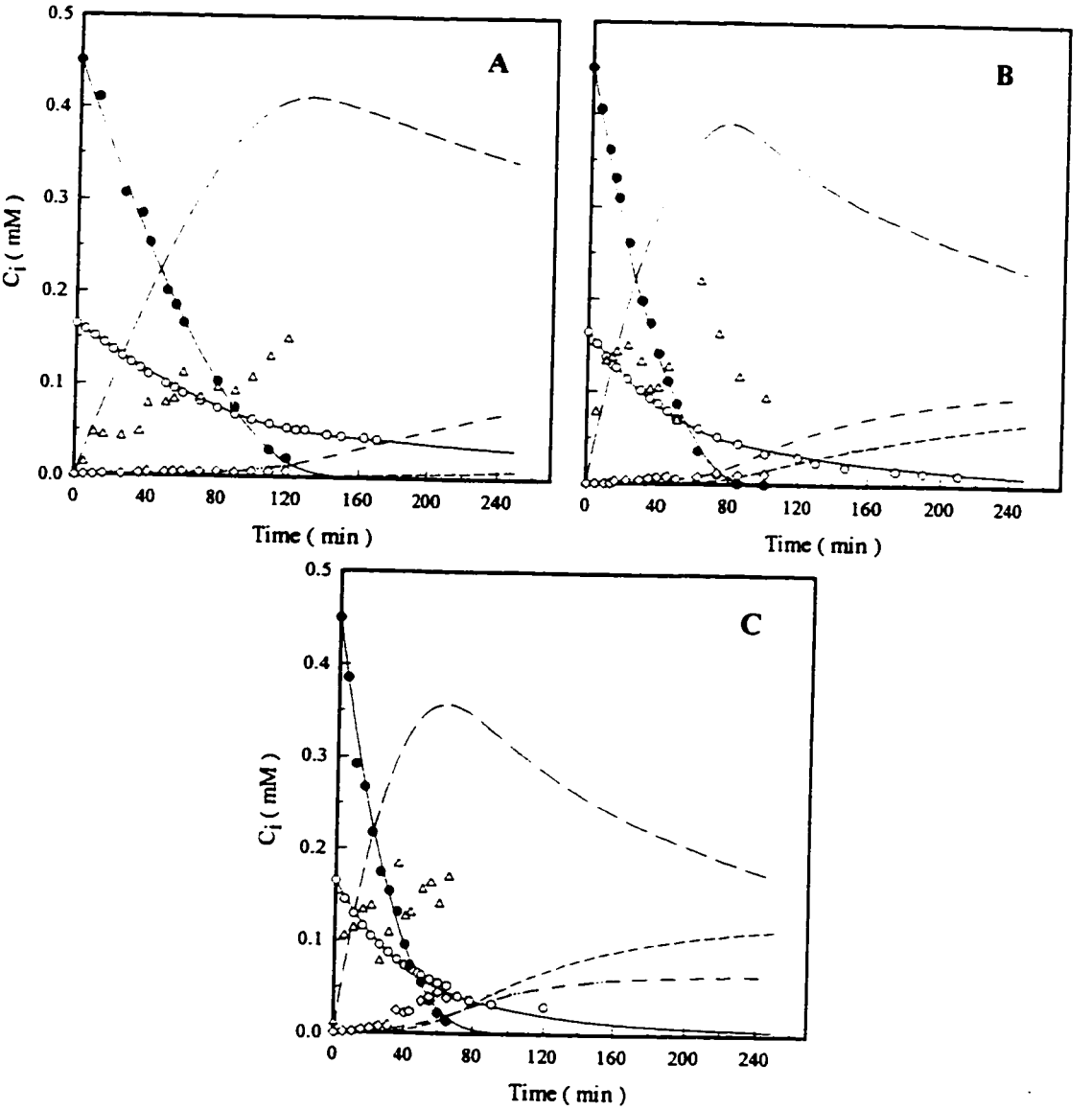


Figure 8-23. Effect of temperature on the time course of the Theorell-Chance Bi-Bi mechanism with three alternate substrates at a constant initial oxygen concentration: (A) 20°C; (B) 33°C; (C) 45°C. Symbols: (O) oxygen; (●) SA; (Δ) DAD; (◊) XV. The lines are the predicted data by Eqs.(8.4)-(8.8): (———) O_2 and SA; (·····) XV; (— — —) DAD; (·—·—·—·) P_2; (– – – –) P_3. The measured concentration of compound DAD was multiplied by 5 for clarity.

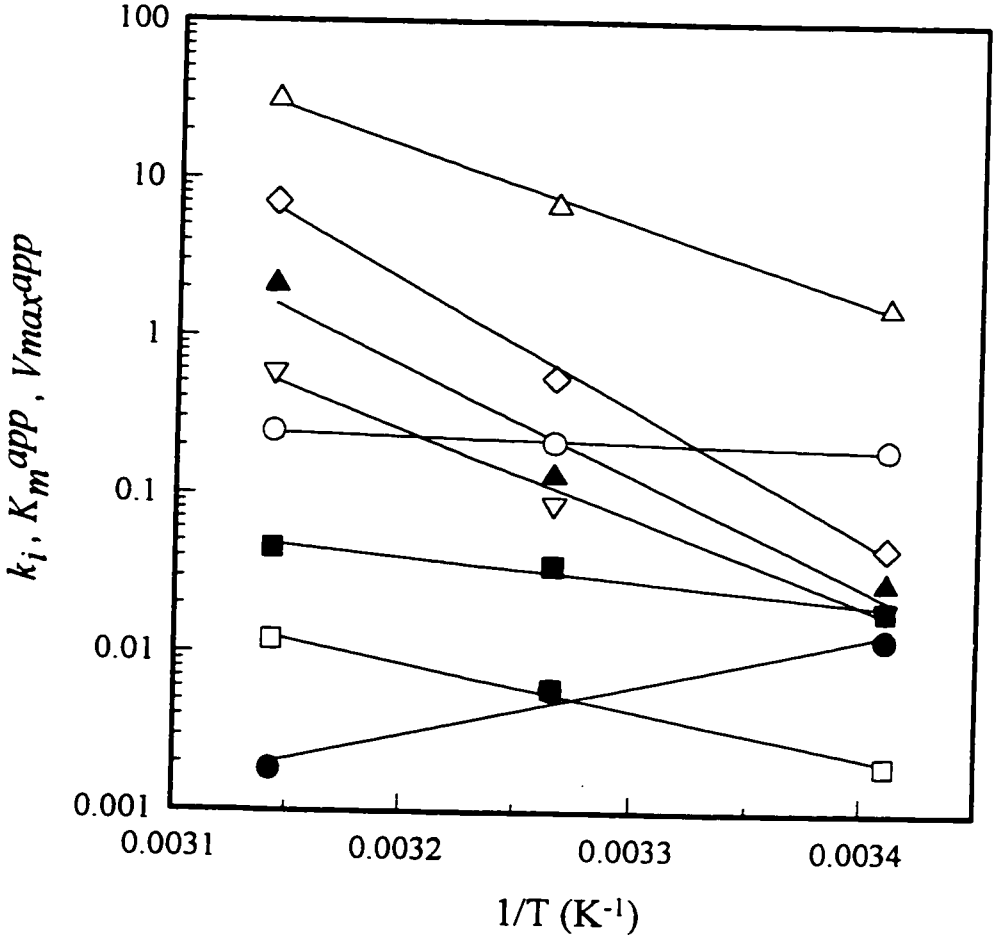


Figure 8-24. Effect of temperature on the respective kinetic constants: (○) k_1; (△) k_{-1}; (□) k_2; (▽) k_3; (◊) k_4; (▲) k_5; (●) $K_{m,St,app}$; (■) $V_{max,app}$.

Table 8-5. Estimated frequencies factors and energies of activation for the respective rate constants based on the transition state theory.*

	k_1	k_2	k_3	k_4	k_{-1}	k_5	k_x	k_d
	[mM^{-1} min^{-1}]				[min^{-1}]			
$k_{0,i}$	3.688	5.571×10^{16}	1.016×10^{17}	2.062×10^{26}	2.199×10^{7}	9.669×10^{21}	2.381×10^{5}	5.122×10^{10}
$E_{i,a}$	4.107	90.000	102.277	152.232	53.272	129.664	62.785	93.288

* energies given in [kJ mol^{-1}]

are given in Table 8-5. A very low activation energy for the rate constant of enzyme-oxygen complex formation indicates that this reaction step is relatively temperature-insensitive. The reported, k_1, for *Rhus* laccase are around 3.6×10^{5} mM^{-1} min^{-1} [Andreasson *et al.*, 1976; Petersen and Degn, 1978]. In this work, k_1 is much smaller, 2.06×10^{2} mM^{-1} min^{-1}; however, it must be noted that the enzyme preparation used in this study was not purified. In addition, the value of this rate constant depends on the arbitrarily chosen value of β.

Initial reaction rates measured in the "closed" system were compared with those obtained from spectrophotometric studies (Section 8.1.5). In the "closed" system, when the temperature was increased from 20°C to 33°C the initial reaction rate increased 1.85 times (as calculated using Eq.(8.2) with the values of the parameters in Table 8-4). However, in the spectrophotometric system, in which the initial oxygen concentration varied with temperature, the same increase in temperature caused 1.5 times increase in the enzymatic activity [Łącki and Duvnjak, 1996a]. A similar effect was observed when the temperature was increased from 33°C to 45°C. Furthermore, according to Table 8-4, the enzyme affinity towards SA decreased with an increase in temperature. This finding contradicts the results obtained from the spectrophotometric studies which showed that $K_{m,SA}$ decreased with increasing temperature (Figure 8-6). However, when the data in Table 8-4 were used to calculate the apparent values of $K_{m,SA}$ according to Eq.(8.3), using the saturation oxygen concentration for a given temperature, the same pattern was observed in the "closed" system as for the spectrophotometric studies. Thus, the results obtained in the 'closed" system proved that the temperature has a much more complex effect on the enzyme characteristic than observed in the spectrophotometric system.

Table 8-4 also shows that the enzyme affinities for the compounds DAD and XV are lower than for SA. This was anticipated because of a two-stage reaction pattern for SA transformation.

8.3.5 Open system

Considering that the enzymatic tests are usually performed spectrophotometrically, it is necessary to specify assay conditions that would minimize the effects of the uncontrolled phenomena (*i.e.*, changes in oxygen concentration with temperature). Since a spectrophotometric cuvette is a system with an unrestricted access to oxygen, it is important to know the changes in oxygen concentration during the spectrophotometric test. Unfortunately, it is difficult to follow the changes in a cuvette and, therefore, an experimental apparatus that resembles a spectrophotometric cuvette, called the "open" system, was used to monitor this phenomenon. Using this apparatus, the effects of the initial enzyme and SA concentrations on the rates of change in the oxygen and SA concentrations were investigated (Figure 8-25). The results predicted by Eqs.(8.4)-(8.8) agreed well with the experimental data for SA, whereas a slight discrepancy was noticed for oxygen. This deviation resulted from predicting the data using estimates from Table 8-4 and not the estimates obtained with the data in Figure 8-25. Because the model is highly sensitive to the values of mass transfer coefficient, k_La, a small variation in the experimental conditions causes the observed deviations between the predicted and experimental results. Figure 8-25 also shows the experimental and predicted data for compounds DAD and XV for the initial SA concentration of 0.1 mM. The difference between the predicted and the actual rates of the DAD and XV transformation resulted because of the rate constants k_3 and k_4, which govern these transformation rates. Both rate constants were estimated using data that pertain only to the rate of oxygen decrease during the enzymatic transformations of DAD and XV (Figure 8-22). Therefore, because the solubility limit of DAD was not included in the model, the concentrations of compound DAD were assumed to be higher than the actual ones. Subsequently, the rate constants k_3 and k_4 were lower than would be obtained if the actual concentrations of DAD were known [Łącki and Duvnjak, 1996c].

According to the results obtained in the open system (Figure 8-25), at higher enzyme or SA concentrations, the oxygen concentration cannot be considered constant during the reaction. This suggests that the spectrophotometric assay should be performed at relatively low enzyme and

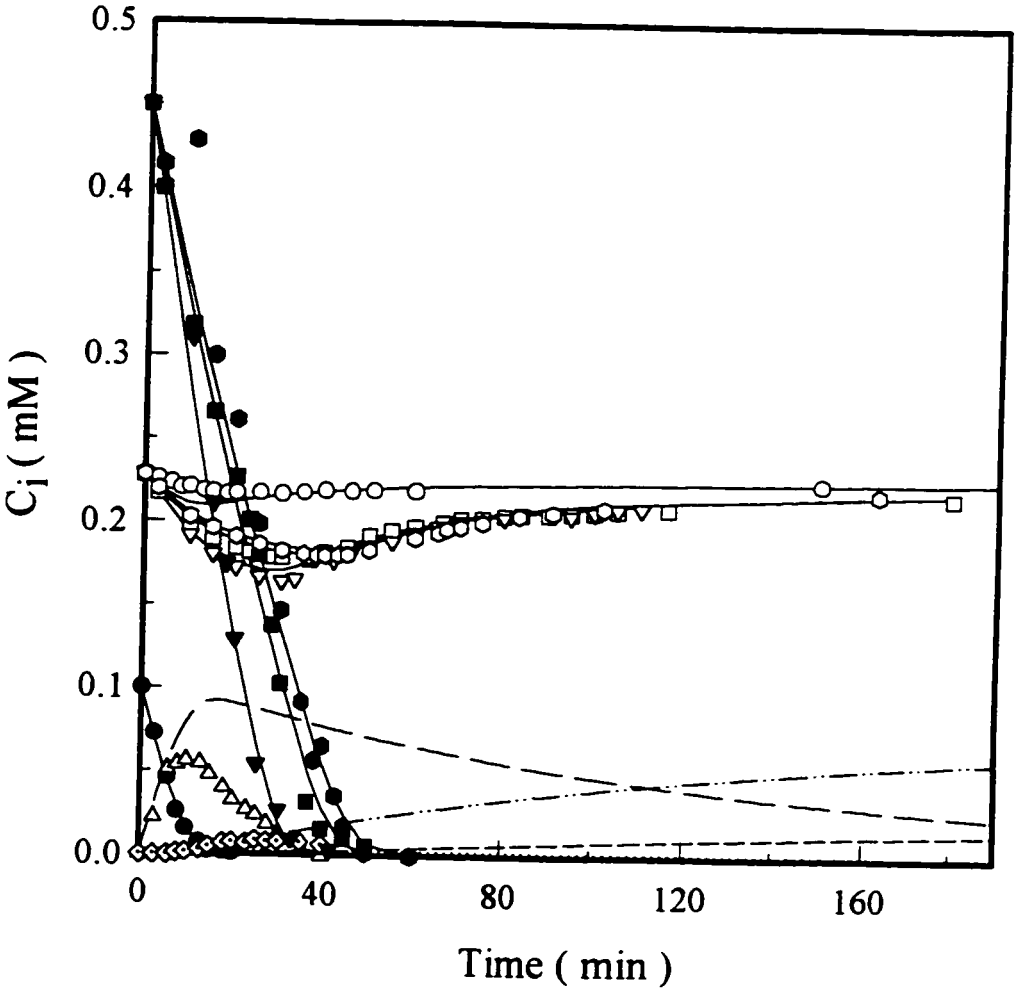


Figure 8-25. Effects of enzyme and sinapic acid concentrations on the concentration-time curves for oxygen (open symbols) and sinapic acid (closed symbols) in the open system at pH 3.5 and 33°C; enzyme added (mL): (⬡) 0.09, (□) 0.1, (▽) 0.14 for SA = 0.45 mM; enzyme added (mL): (○) 0.09, (△) DAD; (◇) XV for SA = 0.1 mM. The lines are the predicted data by Eqs.(8.4)-(8.8): (———) O_2 and SA; (·····) XV; (— — —) DAD; (·—·—·—·) P_2; (– – – –) P_3. The measured concentration of compound DAD was multiplied by 5 for clarity.

SA concentrations. In such cases, the rate of oxygen transfer into the reacting system would be comparable to its utilization rate and the reaction would proceed at a constant oxygen concentration. Furthermore, if the experiment is performed under such conditions, the enzyme kinetic parameters could be calculated using the integrated form of Eq.(8.3) and the transient data from a single run. However, when the oxygen concentration varies during the reaction, the initial reaction rate is higher than the rate of oxygen diffusion into the system, hence the progress curve for SA has to be described by a system of ODEs. In such a case, the integrated form of Eq.(8.3) should not be used to describe the changes in the SA concentration measured, because the rates of SA disappearance are influenced not only by changes in its concentration but also by the varying oxygen concentration. The initial decrease and then increase in oxygen concentration results in a longer linear part of the SA progress curve which leads to the wrong values of the kinetic constants in Eq.(8.3).

When the effect of temperature on enzyme activity is being investigated, the enzymatic assay conditions should be defined to minimize the effect of the change in oxygen concentration on the reaction rate. These conditions were found using Eq.(8.2) and the kinetics parameters from Table 8-4. The computed results for the three considered temperatures are represented as contour plots (Figure 8-26). They indicate that the oxygen concentration strongly affects the reaction rate at higher SA concentrations regardless of temperature. On the other hand, at lower SA concentrations (*e.g.*, at least one order of magnitude lower than that of oxygen concentration), the effect of changing the initial oxygen concentration is almost negligible.

8.4 Modeling of Sinapine Transformation

Considering that sinapine is the most abundant phenolic in CM, it was necessary to develop a model that could predict its enzymatic transformation. The general form of such a model should account for the effects of substrates concentrations, pH of the solution and temperature on the reaction rates. The following sections focused on the development of the model that simultaneously described these effects on the rate of sinapine transformation. The modeling was based on the initial rate measurement data that were collected in this study.

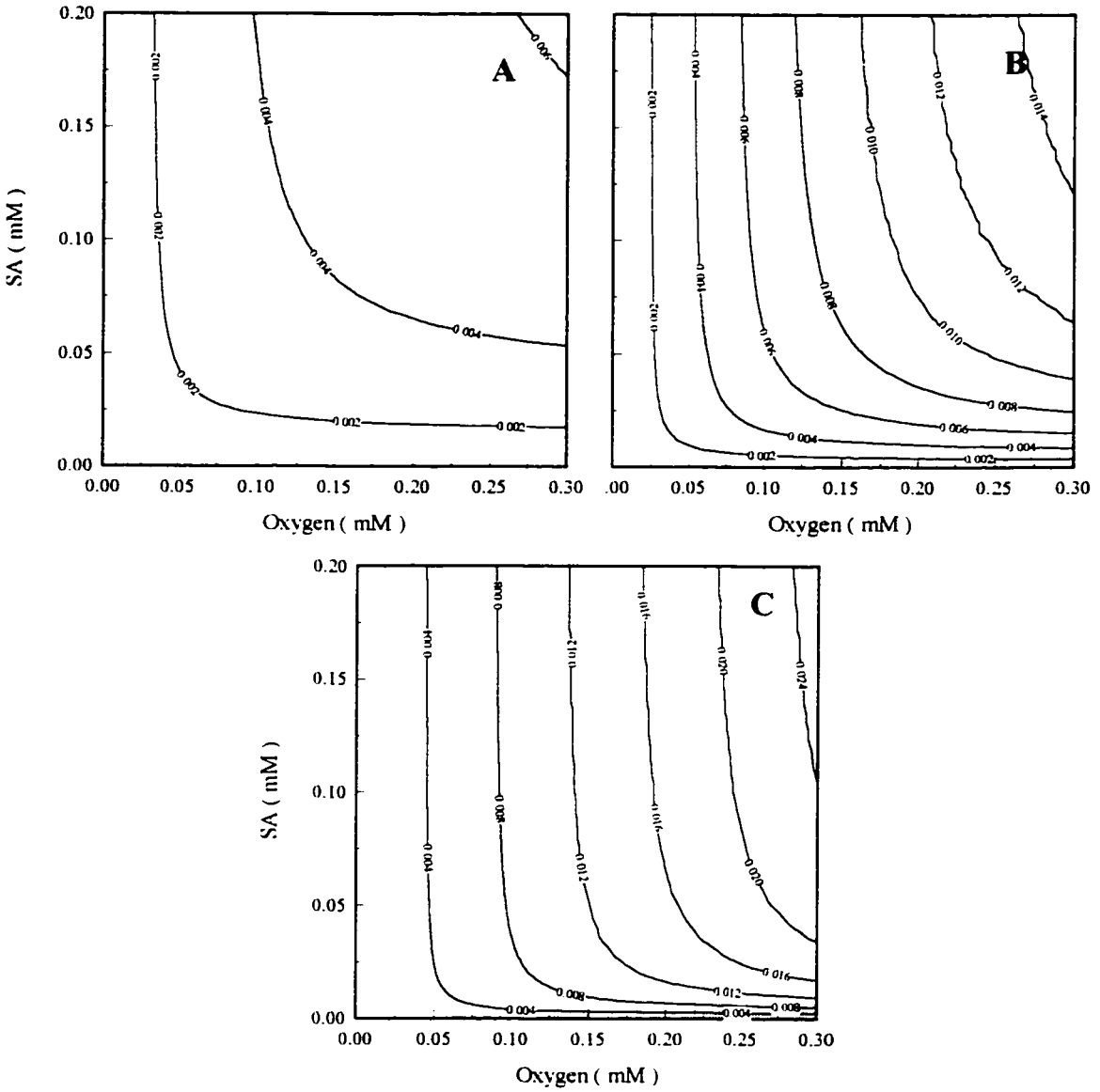


Figure 8-26. Predicted initial reaction rates using Eq.(8.2) for various initial oxygen and sinapic acid concentrations at: (A) 20°C; (B) 33°C; (C) 45°C. The axes and the contours represent the substrate concentrations and the initial reaction rates, respectively.

8.4.1 Development of the model

If it is assumed that the enzymatic transformation of sinapine proceeds according to the same mechanism as the one observed for sinapic acid transformation, *i.e.*, the Theorrel-Chance Bi-Bi mechanism where oxygen [O_2] and sinapine [SIN] combine with the enzyme [E] in an obligate order (Figure 8-27), then, after neglecting the presence of products in the reaction mixture, the initial activity equation derived from a steady state assumption is given by Eq.(8.9) [Segel, 1975].

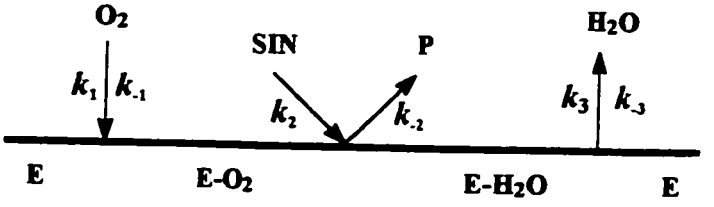


Figure 8-27. Theorell-Chance Bi-Bi mechanism for the transformation of sinapine by polyphenol oxidase from *T. versicolor*.

$$v = \frac{V_{max}}{1 + \frac{K_{m,O_2}}{[O_2]} + \frac{K_{m,SIN}}{[SIN]}\left(\frac{K_{i,O_2}}{[O_2]} + 1\right)} \qquad (8.9)$$

Eq.(8.9) describes the Michaelis-Menten kinetics and is valid for any enzyme concentration for which a linear proportionality of the activity with respect to the enzyme concentration holds. In order to use Eq.(8.9) as a base for a model that predicts the simultaneous effects of the temperature, pH, enzyme and both substrates concentrations on the initial reaction rate of SIN transformation, the following assumptions were made:

(i) The enzyme can be considered a dibasic acid which exists in three forms obtained through the protonation and deprotonation of the active site, with only one form (E^-) being active [Bailey and Ollis, 1986]

$$E^{-2} \xleftarrow{K_1} E^- \xleftarrow{K_2} E^0$$

(ii) The thermal deactivation of the active form of enzyme is described by a simple first order irreversible deactivation mechanism:

$$E_a^- \xrightarrow{k_d} E_d^-$$

(iii) All of the rate constants are defined by the transition state theory:

$$k_i = \alpha_i \left(\frac{k_b T}{h}\right) e^{\Delta S_i^{\ddagger}/R} e^{-E_{i,a}/RT} = k_{0,i} T e^{-E_{i,a}/RT}$$

(iv) The protonation and deprotonation dissociation constants are given by the following equation [Smith and Van Ness, 1987]:

$$\ln K_i = -\frac{\Delta G_i^0}{RT}$$

(v) All of the enzyme kinetic parameters are independent of pH [Bailey and Ollis, 1986];

(vi) The concentration of oxygen in the system (mM) at a given temperature is calculated from its solubility, assuming constant (temperature independent) water density and neglecting the effects of salts concentrations (buffer plus sinapine) on oxygen solubility:

$$[O_2] = \frac{n_{O_2}}{1 - n_{O_2}} \frac{0.21}{18.0} 10^6$$

$$n_{O_2} = e^{-64.2152 + \frac{83.9123}{T/100} + 23.2432 \log \frac{T}{100}}$$

where: n_{O2} is the solubility of oxygen at a particular temperature and at an air partial pressure of 101.325 kPa [Battino, 1981].

The overall model was developed in two steps in which the effects of pH and temperature were evaluated separately, and the relationships obtained were combined to get an overall model.

8.4.2 Effect of pH

It can be shown that using the definitions of the dissociation constants, K_1 and K_2, (assumption (i)) and assuming that the overall enzyme concentration does not change, the concentration of the active form of enzyme is given by Eq.(8.10) [Bailey and Ollis, 1986]:

$$e_0^{\cdot} = \frac{e_0}{1 + \frac{h^{\cdot}}{K_1} + \frac{K_2}{h^{\cdot}}} \qquad (8.10)$$

Since the initial reaction rate is directly proportional to the enzyme concentration, Eq.(8.9) and Eq.(8.10) can be used together to evaluate initial rates at a given temperature for any hydrogen ion concentration.

8.4.3 Effect of temperature

To evaluate the effect of temperature on the reaction rate, the relationships between the enzyme kinetic parameters and temperature have to be known. In addition the enzyme thermal deactivation of the enzyme has to be also taken into account. Here it was assumed that the enzyme deactivates irreversibly, as described by Eq.(8.11).

$$e_a^-(t) = e_o^- e^{(-k_d t)} \tag{8.11}$$

Eq.(8.11), however, cannot be used in the above form in the model based on Eq.(8.9) because it is impossible to directly incorporate any transient effects into the evaluation of the initial rate measurements. Therefore, a different approach that would account for the irreversible enzyme deactivation had to be proposed. If the concentration of the active form of enzyme can be expressed as the time-average concentration during the enzymatic reaction, then at any reaction time, t_r, at which the initial rate is measured, such an average enzyme concentration is given by Eq.(8.12).

$$\left\langle e_a^- \right\rangle = \frac{\int_0^{t_r} e_a^- dt}{\int_0^{t_r} dt} = e_0^- \frac{1 - e^{(-k_d t_r)}}{k_d t_r} \tag{8.12}$$

It should be noted that from a mathematical point of view, such a treatment of the deactivation process, *i.e.*, a constant enzyme concentration at a given temperature, is not different from the one described by the reversible deactivation mechanism which is usually used when evaluating the effect of temperature on enzyme activity [Bailey and Ollis, 1986]. In both cases the enzyme concentration is assumed to be constant during the reaction. Yet, the proposed method is more appropriate in cases when irreversible deactivation takes place.

8.4.4 Combined effects of pH and temperature

If Eq.(8.12) and Eq.(8.10) are combined and substituted into Eq.(8.9), the following expression for the initial reaction rates at a constant temperature and at any pH is obtained:

$$v = \frac{\beta k_3 e_0}{1 + \frac{h^+}{K_1} + \frac{K_2}{h^+}} \frac{1 - e^{(-k_d t_r)}}{k_d t_r} \frac{1}{1 + \frac{K_{m,O_2}}{[O_2]} + \frac{K_{m,SIN}}{[SIN]}\left(\frac{K_{i,O_2}}{[O_2]} + 1\right)} \qquad (8.13)$$

In order to use Eq.(8.13) to predict the initial rate at any temperature, the parameters k_3, k_d, K_1, K_2, K_{m,O_2}, K_{i,O_2}, and $K_{m,SIN}$ had to be expressed as functions of this variable. Using assumptions (iii) and (iv), and expressing the enzyme kinetic parameters as the ratios of the respective rate constants (Table 8-3), the overall model that predicts the initial reaction rates at any temperature, pH, enzyme and sinapine concentrations is given by Eq.(8.14).

$$v = \frac{\beta e_0}{1 + \frac{e^{\Delta G_2^0/RT}}{h^+} + \frac{h^+}{e^{\Delta G_1^0/RT}}} \frac{1 - e^{-\left(t_r k_{0,d} T e^{-E_d/RT}\right)}}{t_r k_{0,d} T e^{-E_d/RT}} \frac{k_{0,3} T e^{-E_{a,3}/RT}}{1 + \frac{\frac{k_{0,3}}{k_{0,1}} e^{-\Delta E_{a,13}/RT}}{[O_2]} + \frac{\frac{k_{0,3}}{k_{0,2}} e^{-\Delta E_{a,23}/RT}}{[SIN]}\left[1 + \frac{\frac{k_{0,-1}}{k_{0,1}} e^{-\Delta E_{a,-11}/RT}}{[O_2]}\right]} \qquad (8.14)$$

For the sake of simplicity for the representation of Eq.(8.14), the oxygen concentration was not expressed as a function of temperature (assumption (vi)). For the parameters estimation non-simplified form of Eq.(8.14), including the effect of temperature on oxygen concentration was used.

8.4.5 Parameter estimation.

The parameters in the Eq.(8.14) were estimated using non-linear regression analysis based on the Levenberg-Marquardt algorithm. However, before any estimation was performed some issues related to the estimation procedure had to be addressed.

First of all, because of the complicated form of the temperature response (many exponential functions) a transformation of the parameters in Eq.(8.14) was performed. The transformation was done according to Eq.(8.15), which represents a recommended parameters transformation [McLean, 1985] that helps to overcome difficulties related to the estimation problem often met in the estimation of the Arrhenius like relationships.

$$k_i = k_i^* e^{\left[-\frac{E_{i,a}}{R}\left(\frac{1}{T}-\frac{1}{T^*}\right)\right]} \tag{8.15}$$

Secondly, the problem related to the lack of any information about the initial values for most of the parameters had to be solved, since very often, the convergence of a non-linear estimation technique depends severely upon the right choice of the initial estimates [McLean, 1993]. There are many possible ways for generating proper initial guesses and these include: obtaining information from other relevant experiments, a grid search, or a so called multistage estimation. The last technique relies upon breaking the data set into groups so that the auxiliary parameters for each group may be determined [McLean, 1993]. To some extent, all three techniques were employed in this work. It can be pointed out that the parameter transformation used in this work (Eq.(8.15)) also was useful in overcoming the initial guess problem. For instance, if the value of a rate constant is known at one temperature, then setting T^* equal to this temperature results in the parameter k_i^* being equal to that known rate constant. Then the initial guess for the respective activation energy can be found by keeping k_i^* constant, and performing the estimation with the number of parameters being reduced by half.

The final procedure used for the estimation of the parameters in the overall model consisted of two steps. In the first step, the data set was broken into groups of results pertaining to the effect of pH at constant temperatures. These groups were used to evaluate the dissociation constants of protonation and deprotonation reactions, K_1 and K_2, at a given temperature, according to the new method developed in this work. In the second step, the effect of temperature was determined following a new approach developed for the purpose of this work. According to this new routine, instead of performing the parameter estimation on the groups of data pertaining to the effect of temperature at constant pH using Eq.(8.14) and then calculating the average value for each of the parameters, the estimation was done using the results obtained from the modeling of the effect of pH at constant temperature.

The principle for the first step was based on the fact that at any given temperature an optimum hydrogen ion concentration (h^+_{opt}) exists for which the initial activity is at its maximum

and is equal to V_{max}^{opt}. Starting with Eq.(8.14) it can be shown that the optimum hydrogen ion concentration, h^{-}_{opt}, is given by Eq.(8.16).

$$h^{-}_{opt} = \sqrt{K_1 K_2} = 10^{-pH^{opt}} \tag{8.16}$$

According to assumption (v), the ratio of the maximum activity at a given pH to the respective activity at the optimum pH is equal to the ratio of initial rates at the respective pH values, regardless of the substrate concentration, and is given by Eq.(8.17).

$$\frac{V_{max}}{V_{max}^{opt}} = \frac{v}{v_{pH}^{opt}} = \frac{1 + 2\sqrt{K_2 / K_1}}{1 + \frac{K_2}{h^{-}} + \frac{h^{-}}{K_1}} \tag{8.17}$$

A similar expression was proposed by Segel [1975], although the concept behind that formula was to provide another method for the evaluation of K_1, and K_2; however, that method required prior knowledge of pH_{opt} as well as V_{max}. After rearranging, Eq.(8.17) can be used to estimate the three unknown parameters, v_{pH}^{opt}, K_1, and K_2 from the initial rate measurement data obtained at constant temperature and various pHs. These parameters were estimated for each temperature (Table 8-6) and used in Eq.(8.17) to predict the effect of pH on the initial activity (Figure 8-28). The predicted data agree fairly well with the experimental results which showed that

Table 8-6. Estimates for the parameters in Eq.(8.17)

T [K]	K_1 [M]	std. Dev.	K_2 [M]	std. Dev.	v_{pH}^{opt} [nkat]	std. dev.
283.2	1.03E-03	1.28E-04	1.01E-05	1.08E-06	1.79E-01	3.27E-03
293.2	4.40E-04	4.63E-05	8.81E-06	8.76E-07	2.45E-01	4.45E-03
303.2	1.95E-03	2.40E-04	3.60E-06	3.54E-07	4.05E-01	6.08E-03
313.2	1.38E-03	9.63E-05	3.07E-06	1.94E-07	0.45171	4.32E-03
323.2	1.45E-03	9.44E-05	1.63E-06	1.10E-07	4.66E-01	3.98E-03
333.2	8.95E-04	9.03E-05	1.41E-06	1.67E-07	4.47E-01	6.73E-03
338.2	4.41E-04	4.73E-05	2.31E-06	2.57E-07	4.15E-01	7.62E-03
343.2	3.01E-04	5.71E-05	2.16E-06	4.84E-07	3.94E-01	1.38E-02
353.2	4.40E-05	7.01E-06	5.28E-06	9.54E-07	2.18E-01	6.09E-03

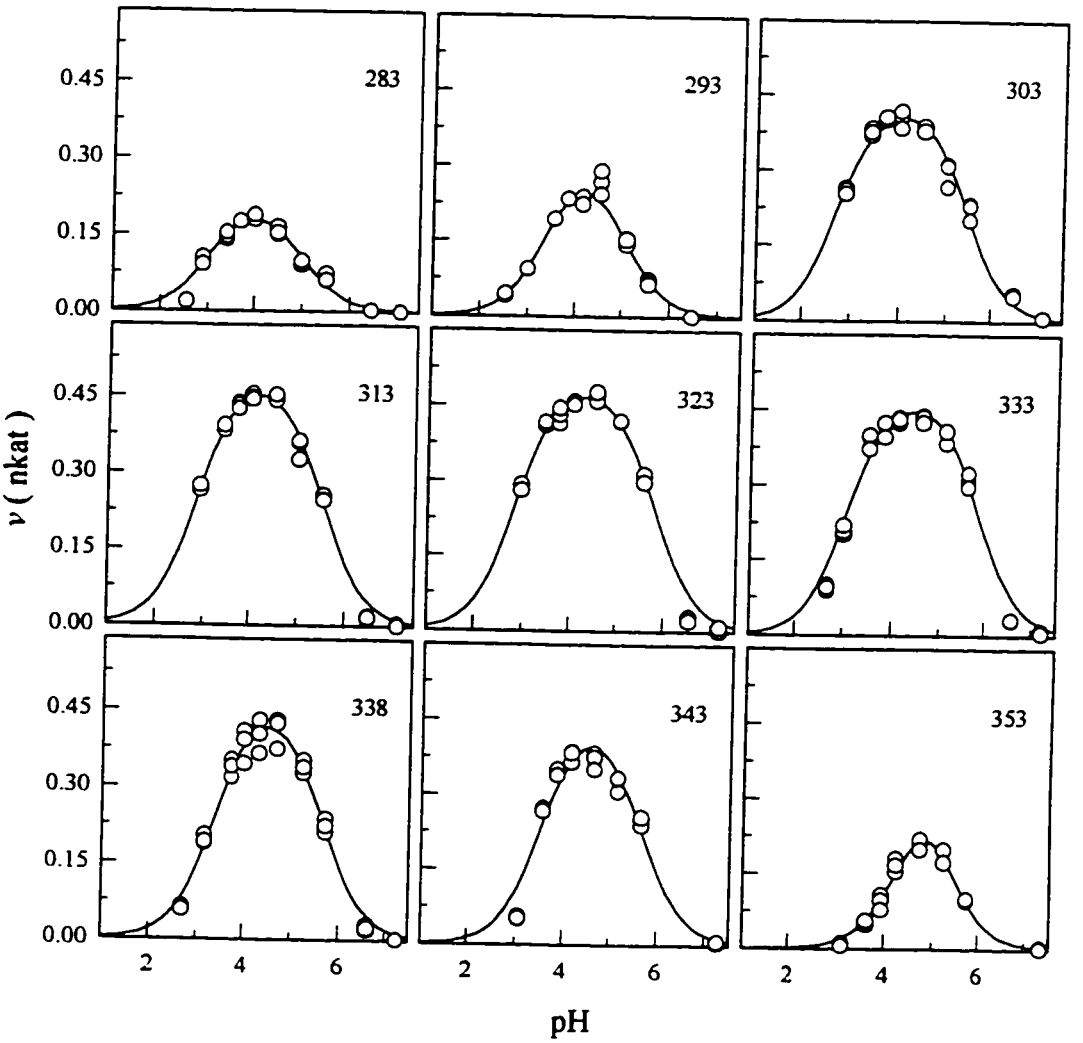


Figure 8-28. Effect of pH on the enzyme activity at various temperatures. Symbols represent experimental data, solid lines are predicted values using Eq.(8.17) with the values of parameters from Table 8-6.

the data from Table 8-6 could be used to obtain a relationship between the dissociation constants and temperature.

As presented in Figure 8-29, the anticipated straight line relationship between the natural logarithm of the dissociation constants values and the reciprocal of temperature, usually obtained when the standard enthalpy of change is temperature independent, was not observed. However, when the dissociation constants were defined using the standard Gibbs energy of reaction, which depends on temperature according to Eq.(8.18), the data presented in Figure 8-29 were adequately described.

$$\Delta G_i^0 = J_i - RT\left(\Delta A_i \ln T + \frac{\Delta B_i}{2}T + \frac{\Delta C_i}{6}T^2 + \frac{\Delta D_i}{2T^2} + I_i\right) \tag{8.18}$$

In order to obtain intuitively acceptable results for the respective dissociation constants outside the range of tested temperatures, the number of parameters in Eq.(8.18) was decreased by setting ΔA and ΔD equal to zero. This resulted in a negligible decrease in the accuracy of fit, but allowed more reasonable predictions of K_1 and K_2, thus increasing the generality of the proposed model. Consequently, the following relationship between dissociation constant and temperature, Eq.(8.19), was used in the overall model:

$$K_i = e^{\left(\frac{-J_i}{RT} + \frac{\Delta B_i}{2}T + \frac{\Delta C_i}{6}T^2 + I_i\right)} \tag{8.19}$$

The estimated values of the parameters in Eq.(8.19), for each of the dissociation constants, are given in Table 8-7. These values can also be used to calculate enthalpy and entropy changes for protonation and deprotonation reactions involving the native form of the enzyme.

Table 8-7. Parameter estimates for the relation between dissociation constants, K_1 and K_2, and temperature

	J	ΔB	$\Delta C*10^3$	I
K_1	-1839322.4	15.272	-51.561	-2261.327
K_2	1721477.4	-13.756	45.240	2062.740

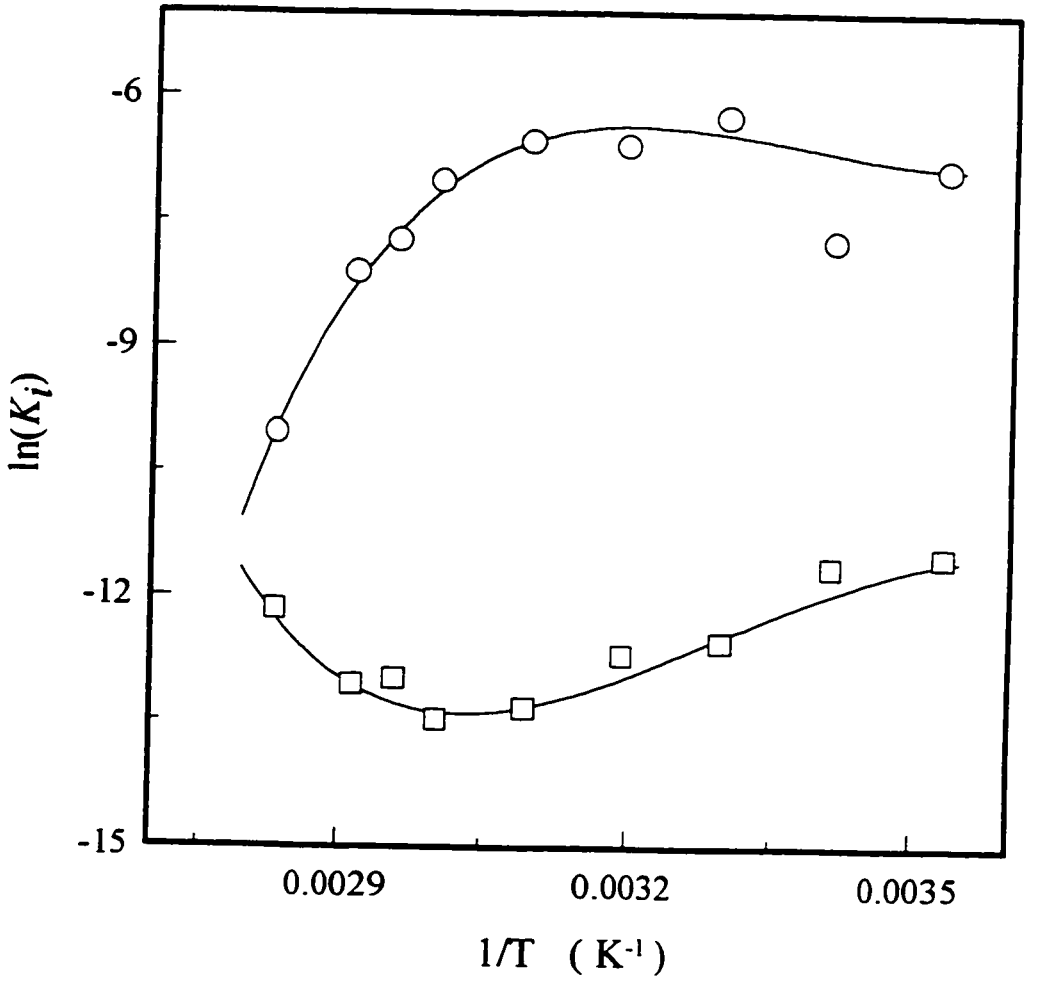


Figure 8-29. Relationship between dissociation constants K_1 (O) and K_2 (□) and temperature. Solid lines are predicted data by Eq.(8.19).

Although from a thermodynamic point of view the deviation from a straight line might be expected [Smith and Van Ness. 1987], the deviation shown in Figure 8-29 seems to be rather large, indicating that the model used to describe the effect of pH on the initial activity is an oversimplification of the actual situation. Nevertheless, Eq.(8.19) was used in the final form of the proposed overall model describing initial rates of SIN transformation.

Having established K_1 and K_2 as functions of temperature, the next step (step two in the proposed overall parameter estimation procedure) was to find the initial estimates for the other parameters in Eq.(8.14). The values for the deactivation energy, E_d, and frequency factor, k_{od}, were taken from the results obtained in the enzyme deactivation experiments. The respective values for the rate constants k_1, and k_{-1}, were taken from relevant experiments regarding the enzymatic transformation of sinapic acid. The initial values of remaining parameters were educated guesses. They were obtained using the new approach proposed in this work, that allowed the estimation of the parameters using a special data set created by applying the estimation results regarding the runs at constant temperature (Table 8-6). This new approach introduces a corrected initial reaction rate, Y_c, that can be obtained by combining Eqs.(8.14) and (8.17). This new quantity given by Eq.(8.20) and Eq.(8.21) can be calculated using data from Table 8-6 (Eq.(8.20)), and can also be expressed as a function of temperature using all of the parameters of interest (Eq.(8.21)).

$$Y_c = v_{pH}^{opt}\left(1 + 2\sqrt{K_2/K_1}\right) \tag{8.20}$$

$$Y_c = \frac{1 - e^{-\left(t_r k_{0,d} T e^{-E_d/RT}\right)}}{t_r k_{0,d} T e^{-E_d/RT}} \cdot \frac{\beta e_0 k_{0,3} T e^{-E_{a,3}/RT}}{1 + \frac{\frac{k_{0,3}}{k_{0,1}} e^{-\Delta E_{a,13}/RT}}{[O_2]} + \frac{\frac{k_{0,3}}{k_{0,2}} e^{-\Delta E_{a,23}/RT}}{[SIN]}\left[1 + \frac{\frac{k_{0,-1}}{k_{0,1}} e^{-\Delta E_{a,-11}/RT}}{[O_2]}\right]} \tag{8.21}$$

The data calculated using Eq.(8.20) (Figure 8-30) can be used to estimate the parameters in Eq.(8.21). The estimates obtained by this method could be more accurate since the effect of temperature on the optimum pH of reaction is included in the data. However, after performing the

initial estimation runs using this set of data, it was found that the additional information concerning the effect of substrate concentration on the initial activity at constant temperature was required (Figure 8-30) to narrow the parameter space and to obtain the more realistic values of the estimates. The estimates obtained using this expanded set of data are given in Table 8.8. They were used to predict the effect of temperature on the Y_e for different sinapine concentrations (Figure 8-30).

Table 8.8. Estimated frequencies factors and energies of activation for the respective rate constants based on transition state theory.[#]

	k_1	k_2	k_{-1}	k_3	k_d
		[mM^{-1} s^{-1}]		[s^{-1}]	
k_0	0.1008	3.7181	$8.748\ 10^5$	$1.241\ 10^9$	$7.079\ 10^9$
E_a [kJ mol^{-1}]	4.107	10.113	53.379	64.087	105.622

[#] enzyme concentration given by $e_0 = \beta \times v_{enz}/V_{sys} \times dil$; v_{enz}= 0.1 mL; $\beta = 1.25 \times 10^{-4}$ mmol/mL of enzyme stock solution; dil = 0.05.

The results obtained showed that the newly proposed method for finding the initial guesses of the parameters resulted in realistic values of the respective parameters. Furthermore, the estimation runs performed on the whole data set (effect of pH and temperature together) did not result in a change of the estimates from their initial values. Consequently, the parameters given in Table 8-7 and Table 8.8 were used to calculate the effects of pH and temperatures on the enzyme initial activity using Eq.(8.14). The obtained results are presented in the form of an activity-surface plot (Figure 8-31).

To check the adequacy of the proposed model, the predicted results were plotted against the experimental ones (Figure 8-32). The random distribution of the results indicated that the proposed model predicts experimental data fairly well, and its accuracy does not depend on the magnitude of the initial rate. Furthermore, the lack of any visible functionality in the residuals with respect to both pH and temperature (Figure 8-33) indicates that the model is not restricted to any particular range of these variables.

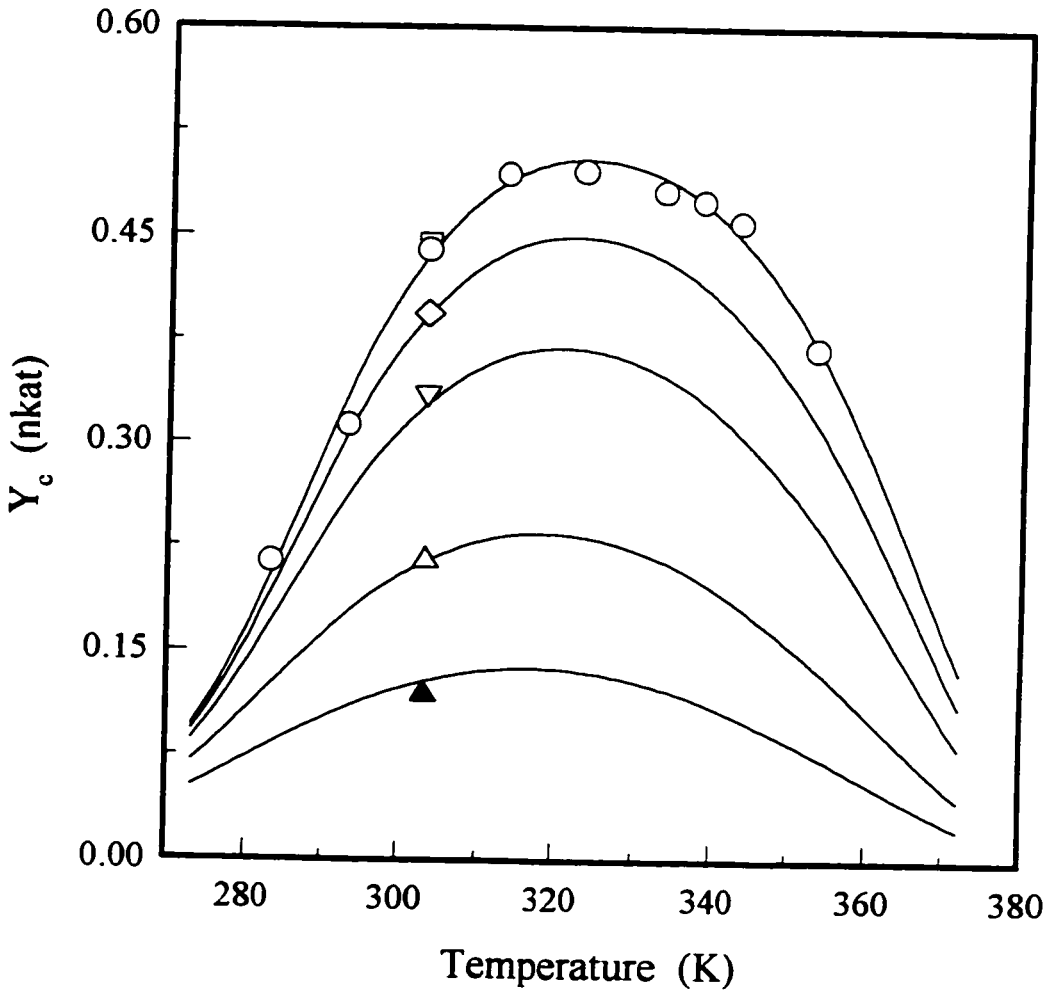


Figure 8-30. Relationship between Y_c and temperature. Solid lines as predicted by Eq.(8.21). Sinapine concentration: (O) 0.116 mM, data calculated from Table 8-6; (□) 0.116 mM; (◊) 0.087 mM; (▽) 0.058 mM; (Δ) 0.028 mM; (▲) 0.014 mM, data obtained at pH 3.7.

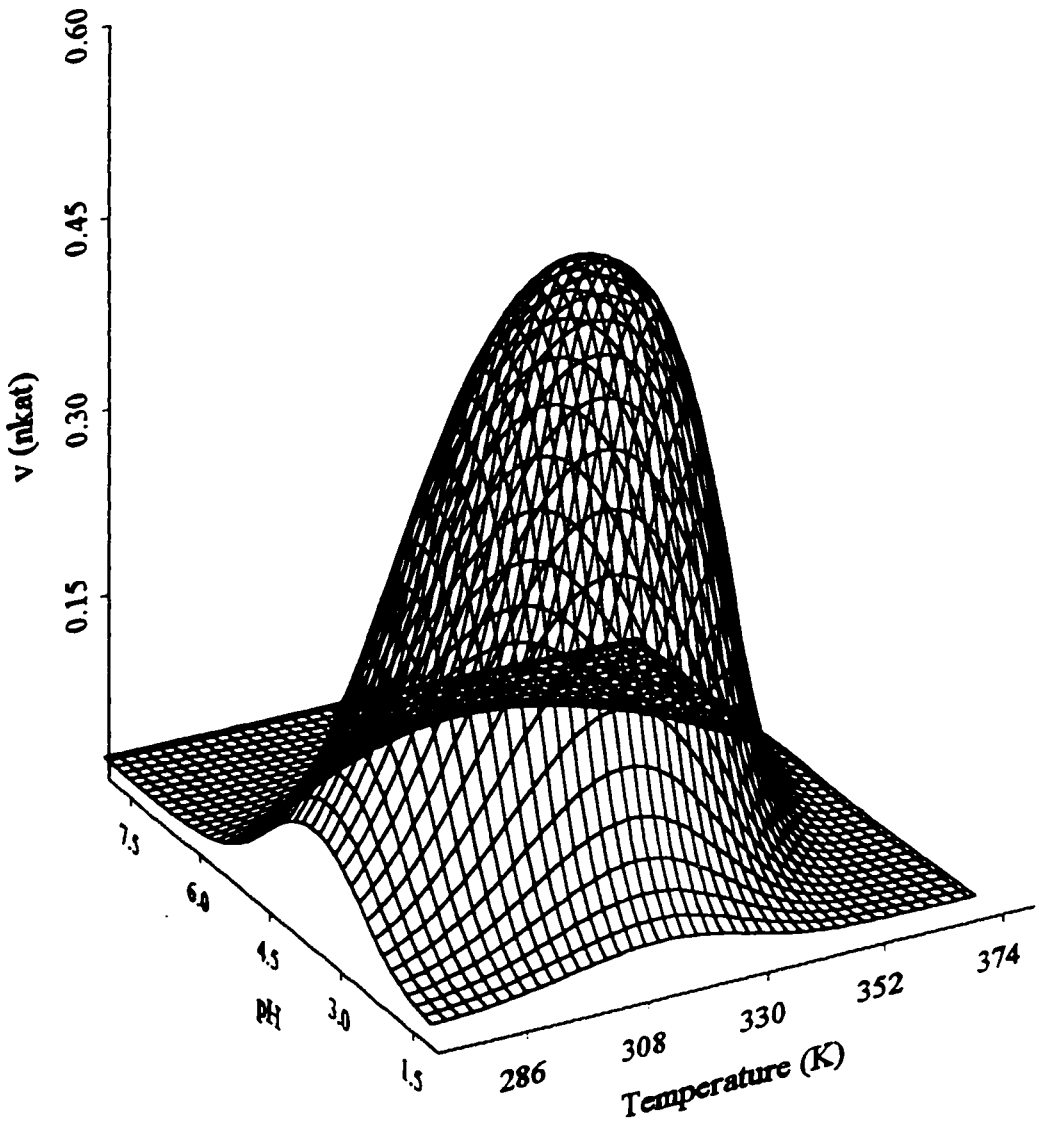


Figure 8-31. Effects of temperature and pH on the sinapine oxidase activity in the model system predicted by Eq.(8.14) for sinapine concentration of 0.116 mM; enzyme dilution 20, and reaction time 10 min.

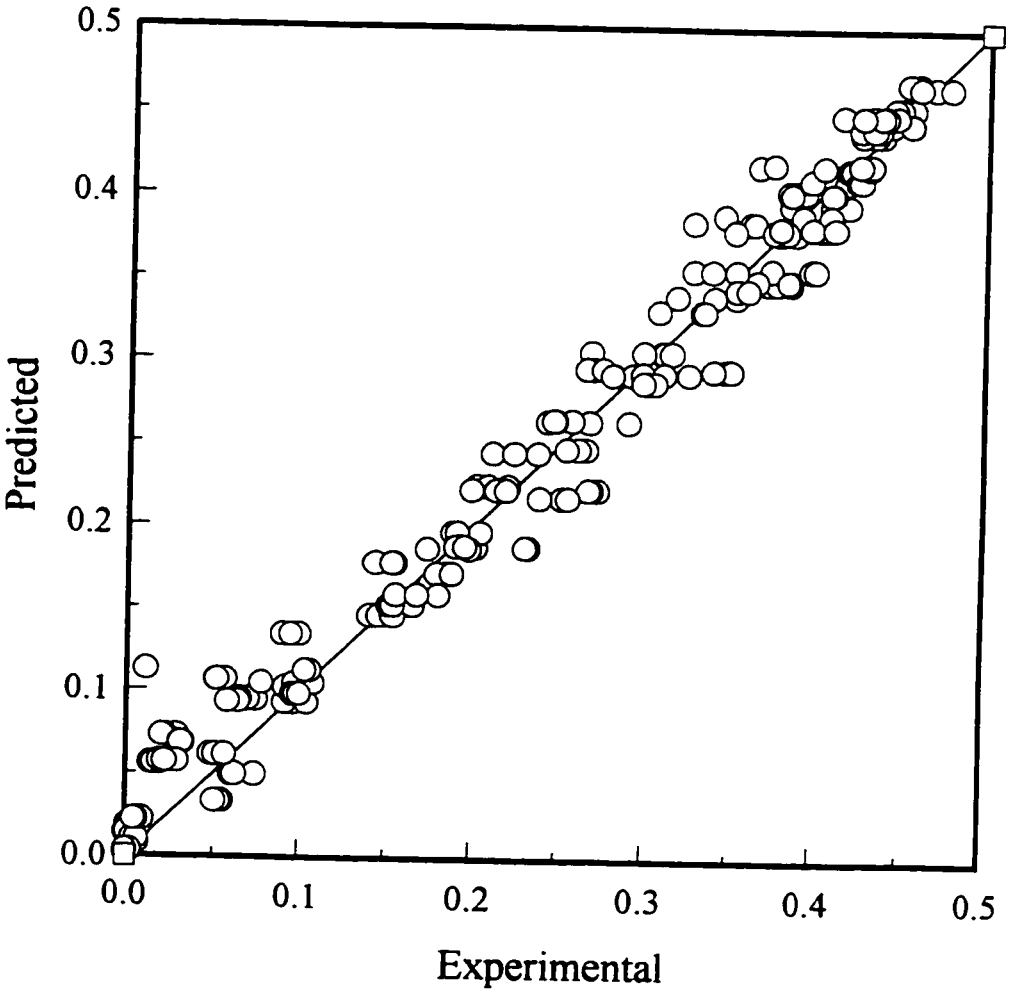


Figure 8-32. Comparison between the experimental activities and those predicted by Eq.(8.14).

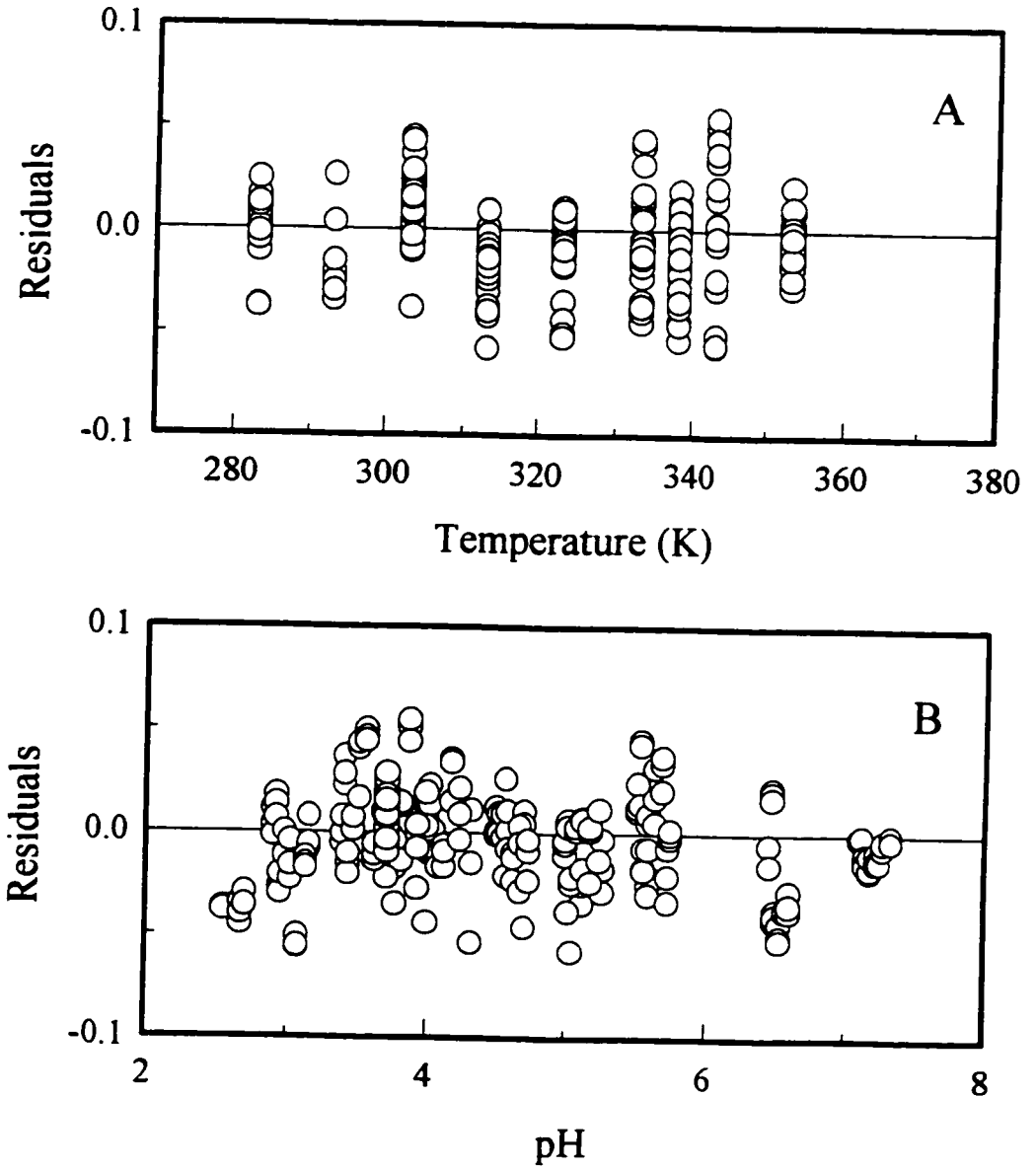


Figure 8-33. Plots of residuals for the proposed model, with respect to: (A) temperature; (B) pH.

8.4.6 Effect of temperature on the optimum pH of reaction

Another interesting result from the study of the effects of pH and temperature on the initial reaction rate was obtained. Since the quantities K_1 and K_2 are functions of temperature, according to Eq.(8.16), the optimum pH for an enzymatic reaction has to be temperature dependent. Combining Eqs.(8.16) and (8.19) yields Eq.(8.22), which is a general expression for the pH at which the reaction rate (at any given temperature) will be the highest. This equation is valid for any enzyme that can be considered a dibasic acid (assumption (i)). Figure 8-34, a contour plot of activities extracted from Figure 8-31, shows how the temperature of the reaction affects the optimum pH. This result implies that the reported optimum conditions for the enzymatic reaction, expressed in terms of optimum pH and temperature, should be closely examined since they are highly correlated. In other words, the optimum conditions for the enzymatic reaction should be established from at least two sets of experiments that are focused on the evaluation of the effect of one variable (pH or temperature) at a fixed level of the other.

$$\mathrm{pH}_{opt} = -\log\sqrt{K_2K_1} = -0.217\left[\begin{array}{l} -\dfrac{J_1+J_2}{RT} + (\Delta A_1 + \Delta A_2)\ln T + \dfrac{T}{2}(\Delta B_1 + \Delta B_2) + \\ \dfrac{T^2}{6}(\Delta C_1 + \Delta C_2) + \dfrac{\Delta D_1 + \Delta D_2}{2T^2} + I_1 + I_2 \end{array}\right] \qquad (8.22)$$

With the help of Eq.(8.14) the optimum pH and temperature for the enzymatic transformation of SIN were calculated to be 4.25 and 50°C, respectively. A detailed experimental work confirmed these results. However, at a constant oxygen concentration (no effect of temperature on the oxygen solubility) the optimum reaction temperature, calculated using Eq.(8.14), would be 60°C. Consequently the optimum pH would change to 4.4 (Eq.(8.22)) which is shown in Figure 8-34.

8.4.7 Dynamics of sinapine transformation

Since, from the engineering point of view, the dynamics of the SIN transformation is of interest, the ability of the proposed model to predict the changes in the SIN concentration with time was investigated. When the changes in the oxygen concentration in the system are negligible, the integrated form of Eq.(8.9) can be used to obtain a progress curve for sinapine. On the other hand,

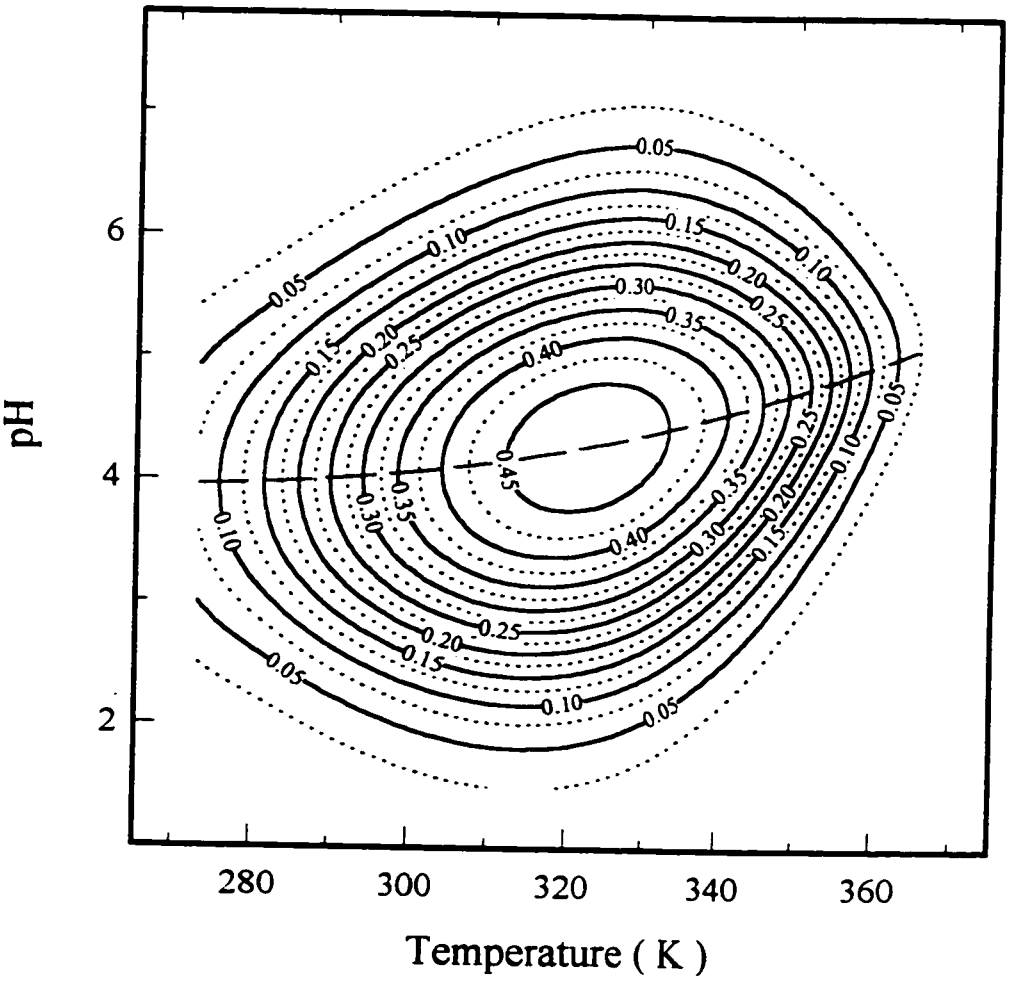


Figure 8-34. Effect of temperature on the optimum pH of enzymatic reaction (dashed line). Constant activity contours as predicted by Eq.(8.14). Activity expressed in nkat/mL_{sys}.

when the oxygen concentration changes during the course of the reaction, the changes in the SIN and oxygen concentrations have to be described by a system of ordinary differential equations (ODE). Such a system of ODE (Eq.(8.23)) can be obtained from Eq.(8.9) by expressing the initial activity as the change in the concentration of a given substrate with time. The factor *m* in Eq.(8.23) accounts for the stoichiometry of the reaction, and denotes the number of molecules of SIN that have to be oxidized to reduce a molecule of oxygen.

$$v = -\frac{d[SIN]}{dt} = -\frac{1}{m}\frac{d[O_2]}{dt} \tag{8.23}$$

To check the model prediction for various conditions of the reaction, a series of experiments were designed to study the effect of the substrates and enzyme concentrations, pH and temperature on SIN transformation in the "closed system". The data obtained are shown in Figure 8-35 A-D. To predict the progress curves for both substrates, SIN and oxygen, the information regarding the stoichiometry of reaction was required. It was expected that the factor *m* would be the same as for the sinapic acid transformation and equal 4 [Łącki and Duvnjak, 1996c]. However, it was observed (Figure 8-35) that according to the experimental results the ratio of the rates of SIN and oxygen disappearance varied from 2.73 to 3.9, depending on the reaction conditions. Since all of these results were smaller than the expected value of 4 one could assumed that the reason for this may be associated with the presence of additional enzymatic reactions involving the products of SIN transformation and oxygen. The existence of these additional reaction steps was indirectly shown in Figure 8-17. Changes in the concentration of two such products, S3 and S7 are presented in Figure 8-35. Because of the lack of actual standards they were calculated using SIN standard curve and, therefore, they should be considered only as relative concentrations. The existence of the reactions involving these compounds and oxygen is documented in Figure 8-35 B, which shows that the rate of oxygen disappearance remained almost constant even after all of the SIN was transformed, and coincided with the disappearance of S7. This could suggest that the affinity of the enzyme towards these new substrates, at least under the experimental conditions discussed, is considerable. If this explanation is correct then it can be stated that the rate of oxygen utilization in the considered tests is greater than the one predicted by Eq.(8.23) because it is a

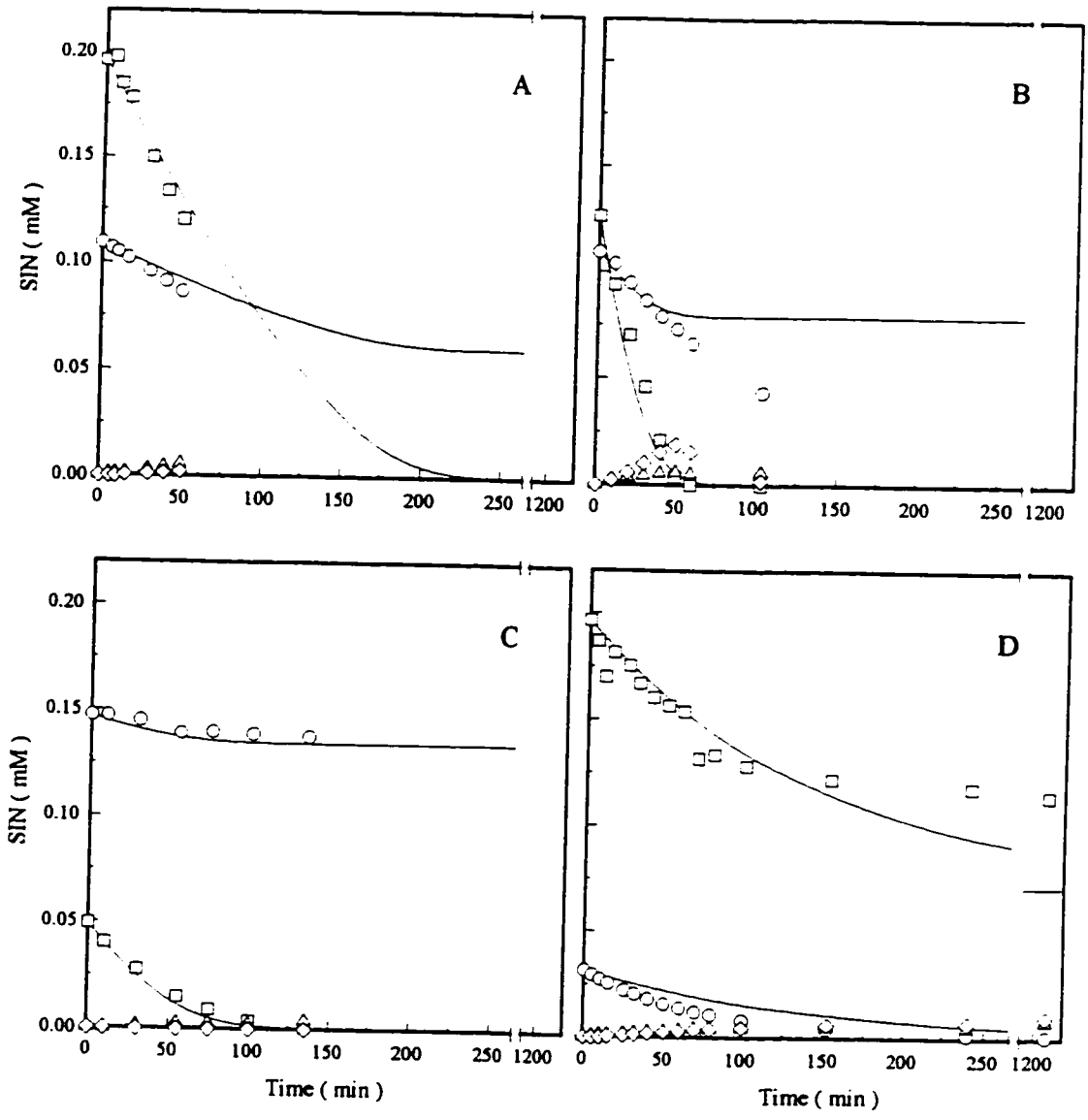


Figure 8-35. Effects of temperature, pH, enzyme, sinapine and oxygen concentrations on the concentration-time curves for oxygen (O), sinapine (□), and compounds S3 (Δ) and S7 (◊). Solid lines represent the predicted data using Eqs.(8.23): (A): [SIN] = 0.195 mM, $[O_2]$ = 0.11 mM, v_{enz} = 0.1 mL, pH 4.5, 15°C ; (B): [SIN] = 0.125 mM, $[O_2]$ = 0.11 mM, v_{enz} = 0.15 mL, pH 5.5, 45°C; (C): [SIN] = 0.05 mM, $[O_2]$ = 0.147 mM, v_{enz} = 0.1 mL, pH 3.1, 15°C; (D): [SIN] = 0.195 mM, $[O_2]$ = 0.0312 mM, v_{enz} = 0.2 mL, pH 4.5, 30°C.

result of the multiple reactions. From Figure 8-35 it is evident that, depending on the reaction conditions, the effect of the additional reactions on the rate of oxygen utilization can be minimized. For example, the formation of compound S7, that appears after the compound S3 is produced, is suppressed by low reaction temperatures (Figure 8-35 A and C). In both of these cases a ratio of the rates of SIN and oxygen disappearance, calculated from the experimental data, is much closer to 4 then in the case of higher temperatures (Figure 8-35 B and D) at which compounds S3 and S7 are noticed much earlier and in greater quantities. The fact that the rate of formation of compound S7 is strongly temperature dependent could indicate that its formation may be governed by the thermolysis of the compound S3. A similar phenomenon was encountered during the enzymatic transformation of sinapic acid. Consequently, at low reaction temperatures, the rate of oxygen utilization represents only the transformation of SIN, and is four times lower than the rate of SIN disappearance. Considering all this, the progress curves for sinapine and oxygen, using Eq.(8.23) was calculated with the values of the respective rate constants from Table 8.8 and the stoichiometry coefficient m equal to 4. The predicted results were compared with the experimental ones for four reaction conditions (Figure 8-35). Except for the limitations related to the presence of additional reactions, the model seems to predict the effect of temperature, pH, enzyme and substrates concentration in a reasonable manner. However, a more detailed analysis related to the investigation of the additional reactions should be performed in order to fully describe this enzymatic system.

Chapter 9

Canola meal system

The upgrading of canola meal (CM) by decreasing its phenolics content was considered. A new enzymatic method was investigated using a commercial canola meal and the polyphenol oxidase from *T. versicolor*. In this chapter, the results obtained from this work are presented and discussed.

9.1 Physico-chemical properties of canola meal

Commercial CM is composed of hulls and cotyledons. Although the hulls are not a desirable part of the meal and should be removed during the industrial processing of the canola seeds, visual examination of the meal used in this study revealed that the hulls were still present in a significant quantity. Attempts to separate them from the cotyledons were not successful and, therefore, the meal was treated as a uniform commodity, even though the composition of the hulls and cotyledons is different.

9.1.1 Physical properties of canola meal

For the purpose of this work two physical properties of CM, water uptake and particle size distribution, were considered.

The water uptake of the commercial meal, defined as a number of mL of water retained by one gram of the meal under processing conditions, was 2.7 mL/g. This is an equivalent to the meal moisture content of 73.0% (w/w) on wet basis. By contrast, the methanol uptake was only 0.3 mL/g.

Table 9-1. Particle size distribution of commercial canola meal.

Diameter [μm]	< 150	> 150	> 355	> 500	> 600	> 710	> 841	> 1000	> 1200	> 1400	> 2000
Mass fraction	0.029	0.147	0.402	0.660	0.759	0.853	0.933	0.963	0.983	0.994	1.0

The particle-size distribution of the meal was obtained by a sieve analysis. The results showed (Table 9-1) that the largest fraction of CM, 51.0% w/w, was made of particles with the diameter within the range of 350 to 600 μm. Considering that the mass of a spherical particle is directly proportional to its radius raised to the third power, it can be concluded that CM was composed of particles with the diameter smaller than 600 μm. The mass average diameter of the particles in commercial canola meal, calculated using the cut off diameters, was 514 μm.

9.1.2 Composition of canola meal

To determine the composition of the commercial meal, the meal was analyzed for several components including moisture, proteins, total available carbohydrates, oil, phytic acid, and phenolics. The results obtained, together with the literature values, are displayed in Table 9-2.

The moisture and oil contents of the meal, 8.0% and 2.0% (w/w), respectively, were typical for the commercial meals. The presence of residual oil indicates that the industrial process for the oil extraction was not completely effective and could be further improved.

Table 9-2. Composition of the commercial canola meal.

Component	This work	Literature value	Reference
Moisture	8.00 ± 0.10	7.5 - 11.0	Alli and Houde, 1987; Blair *et al.*, 1987
Oil	2.00 ± 0.10	1.0 - 3.0	Shahidi, 1990; Bell, 1993
Protein	35.75 ± 2.10	34.0 - 38.0	Clandinin *et al.*, 1986
Carbohydrates	18.22 ± 0.71	10.0 - 22.0	Gattinger *et al.*, 1990; Clandinin *et al.*, 1986
Phytate	6.0*	3.0-7.0	Clandinin *et al.*, 1986
Phenolics#	2.39 ± 0.11	0.96-2.85	Austin and Wolff, 1968; Blair and Richter, 1984

*as determined by Al-Asheh [1993]
#total, including: SAE, SA, IB-SAE, TAN

The total carbohydrates constituted for 18.8% (w/w) of the meal. Fifty per-cent of them consisted of water soluble sugars, including about 9.0% of monosaccharides/free sugars and 91.0% water soluble polysaccharides. The water insoluble fraction was composed of oligosaccharides, such as cellulose and hemicellulose, which together with lignin are the main components of the meal's cell wall.

The high protein content of the meal, 36.0% (w/w), was in agreement with the reported results. A detailed analysis of this fraction was not performed.

The composition of the meal phenolics (Table 9-3) followed a typical pattern reported in the literature (section 2.2.1). Free SA comprised of 10.0% of all phenolics, whereas sinapine, SIN, represented 71.0% of soluble and insoluble SAE. The insoluble-bound phenolics (IB-SAE), expressed as SA equivalent, accounted for 0.01% (w/w) of canola meal. The tannin (TAN) content of 0.28% (w/w) was also within the range of the reported values.

Table 9-3. Composition of phenolic fraction of commercial canola meal

Phenolic	SAE	IB-SAE	SA	SIN	TAN
Concentration	2.110 ± 0.095	0.012 ± 0.00	0.224 ± 0.006	1.503 ± 0.039	0.281 ± 0.012

9.2 Decrease in phenolics content in canola meal by enzymatic treatment

In this section the general effects of the new enzymatic treatment on the phenolic fraction of CM is presented, and some aspects of the enzymatic treatment regarding products formation are addressed.

9.2.1 Effect of the enzymatic treatment on canola meal phenolics

The ability of the enzyme to decrease the SAE content in canola meal was evaluated in the process based upon a direct addition of the enzyme to the meal-buffer slurry. The effect of such a treatment on the composition of the phenolics fraction of CM is shown in Figure 9-1, where the chromatograms of methanol extracts from untreated, active-enzyme treated and deactivated-enzyme treated meals are compared. Almost a complete disappearance of the peaks representing SA and SIN showed that the treatment with the active enzyme was 99.0% effective. In addition, the

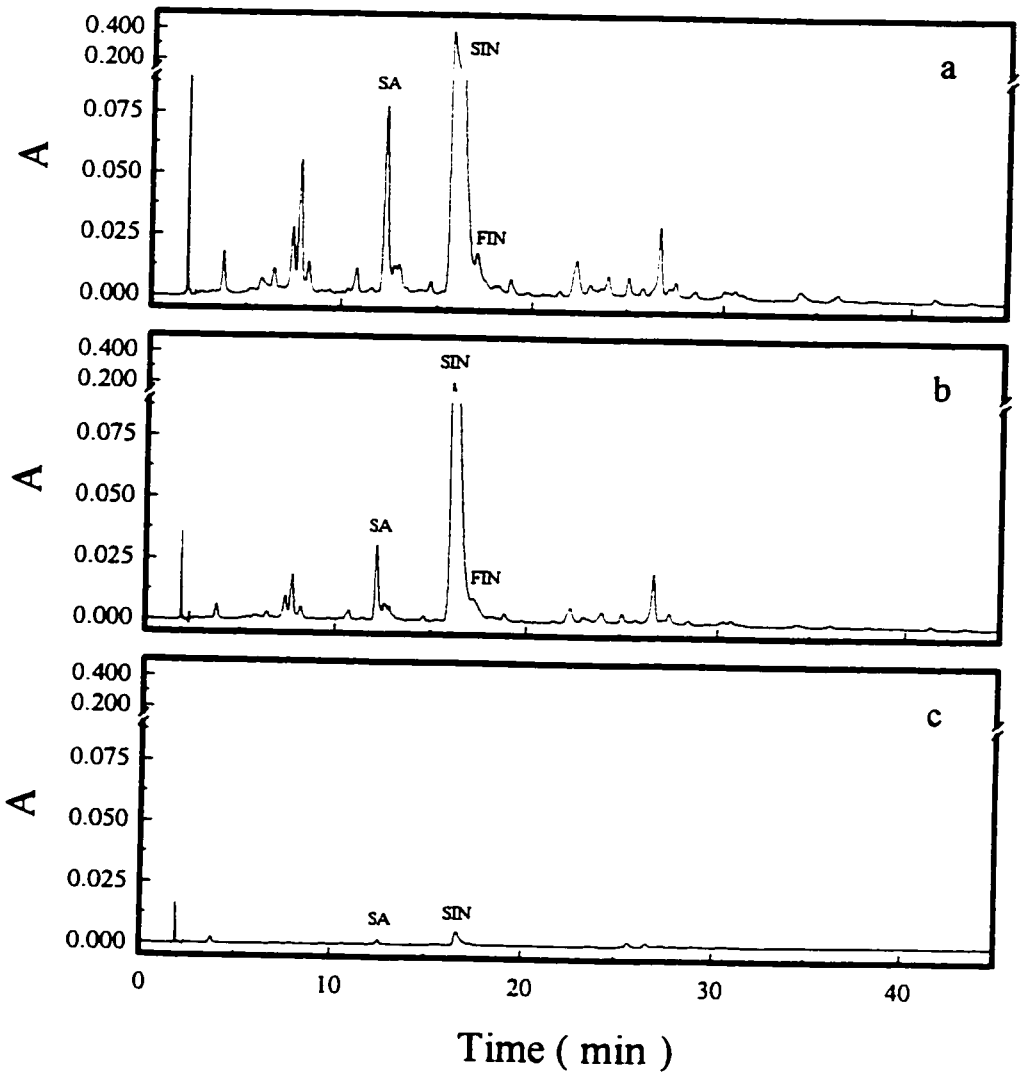


Figure 9-1. Chromatograms of the methanol extract from commercial canola meal: untreated (a); deactivated-enzyme treated (b); active-enzyme treated (c). The treatment of the meal involved the incubation of the meal in buffer-enzyme solution at slurry concentration of 10.0% w/v for 16 hours at 50°C. FIN represents iso-ferulocholine (Appendix C).

concentrations of other phenolic compounds that could be detected using the HPLC separation method (Appendix C) also decreased. The obtained meal had a 0.02% w/w residual phenolic content, which is negligible when the meal quality is considered. Treatment of the meal with the deactivated enzyme resulted in a 50.0% decrease in the SAE content. This decrease was caused by the extraction of phenolics into the continuous phase composed of the buffer solution of the deactivated-enzyme, since no enzymatic reaction took place in this system. This is shown in Figure 9-2 where the chromatograms representing a composition of the continuous phase from the systems with the active and deactivated enzymes are displayed. In the system with the active enzyme, the phenolics were not detected in the continuous phase (Figure 9-2b) because all of them were enzymatically transformed. In the system with the deactivated enzyme, the composition of the continuous phase was the same (relative amounts) as the composition of the phenolic fraction in the untreated CM (Figure 9-2a and Figure 9-1a). This showed that the decrease in SAE concentration in the meal was caused by the extraction step.

The residual content of SIN, 0.02%, in the enzymatically treated meal (Figure 9-1c) showed that the enzymatic process could be diffusion limited at the low concentration of SAE in the meal, *i.e.*, the rate of the phenolic content decrease was equal to the rate of the extraction of SAE from the meal matrix.

The effect of the enzymatic treatment on the composition of the insoluble-bound phenolics, IB-SAE, was also considered. As shown in Figure 9-3, treatment of the meal with either active or deactivated enzyme caused a decrease in the concentration of IB-SAE by 56.0% and 32.0%, respectively. Since SA was the main phenolic acid liberated upon the hydrolysis of this type of meal phenolics, the decrease observed in the active enzyme system could have been expected, especially if the ability of this enzyme to transform several SA derivatives is considered. Yet in this case, the enzymatic reaction would have to take place on the meal's surface because IB-SAE can not be extracted from the meal. On the other hand, the results from the system with the deactivated enzyme showed a 32.0% decrease in the concentration of this fraction. Considering that phenolics may be bound to the meal via proteins, they could be extracted from the meal as protein-phenolic complexes. Even if this hypothesis is true, the larger decrease in IB-SAE observed in the active enzyme system, could be explained by the surface reaction.

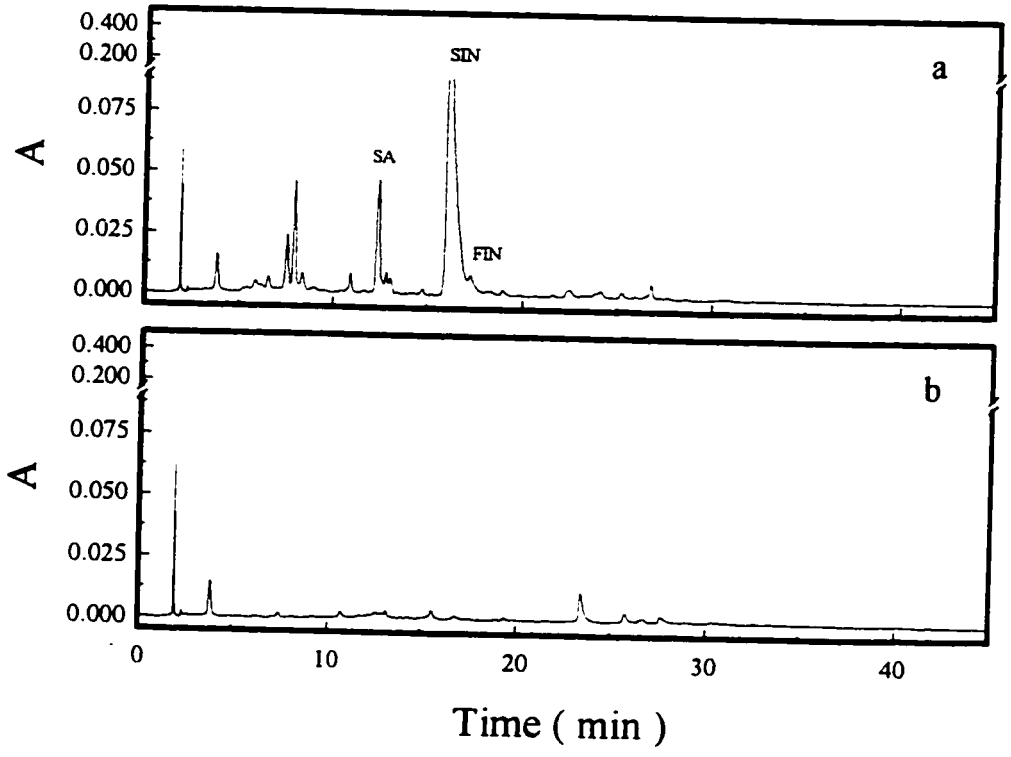


Figure 9-2. Chromatograms of the continuous phase after the treatment of canola meal with: (a) deactivated enzyme; (b) active enzyme. Conditions as for Figure 9-1.

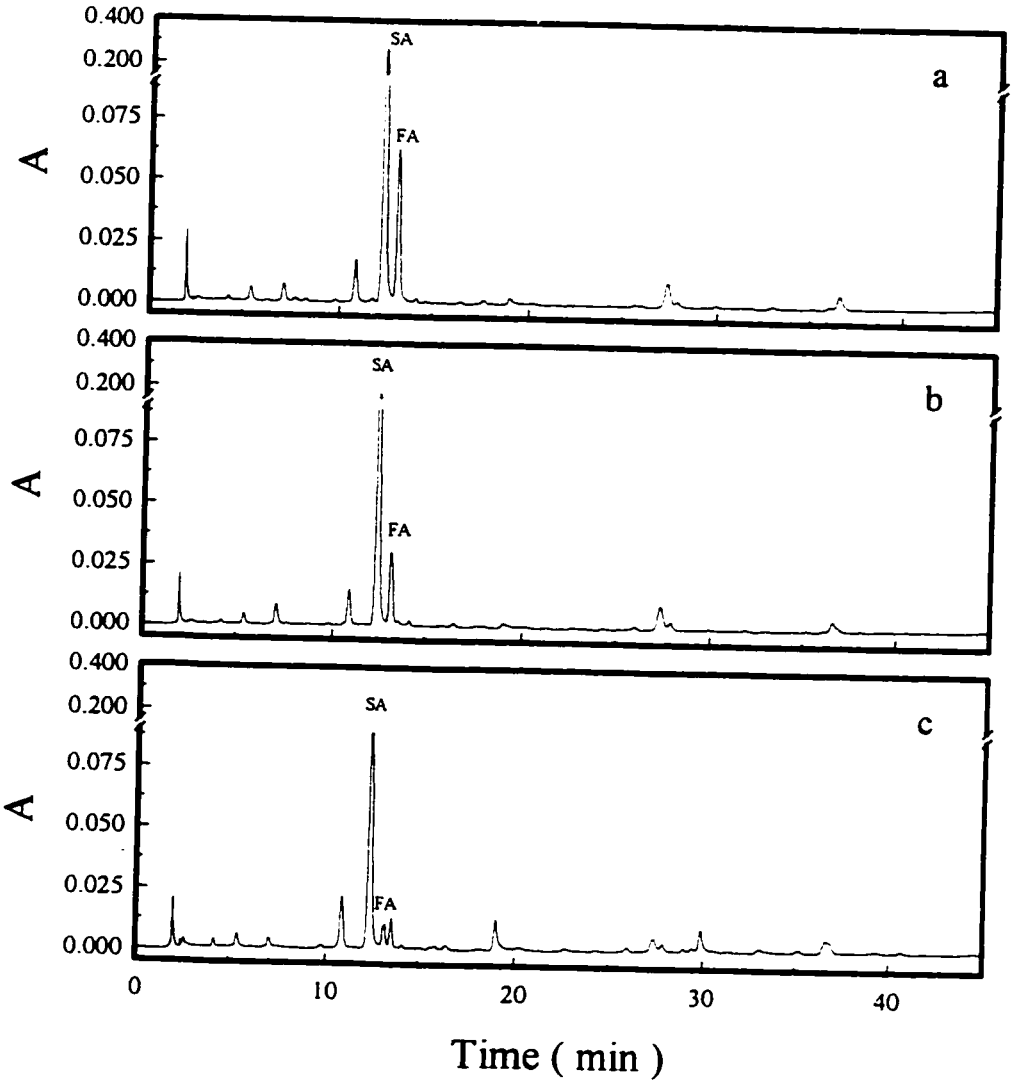


Figure 9-3. Chromatograms representing insoluble-bound phenolics in commercial canola meal: untreated (a); deactivated-enzyme treated (b); active-enzyme treated (c). FA represents ferulic acid. Conditions as for Figure 9-1.

Considering the low concentration of the insoluble-bound phenolics in CM and, therefore, their minimal impact on the meal quality, the effect of the enzymatic treatment at different process conditions on this fraction was not evaluated in this work.

9.2.2 Products formed during the enzymatic treatment of canola meal

Considering that it is desired to use the treated canola meal as a source of nutrients in live-stock diets, it was necessary to determine the products that were formed during the enzymatic treatment. Therefore, the methanol extract of the enzyme-treated meal was analyzed using HPLC with the Photo Diode Array (PDA) detector for the presence of new compounds that originated from the enzymatic treatment. The data obtained in these tests were analyzed according to a procedure for analysis of PDA data from reacting systems (Appendix D) that was developed in this work. The data obtained after 15 min-, 1 hour- and 12 hour-long treatments of the meal with the active enzyme at 50°C and at a meal-buffer slurry concentration of 10.0% (Figure 9-4) were considered. In addition, samples collected from the continuous phase at the respective times of the process were also analyzed for the presence of new compounds (Figure 9-5). The obtained results showed that more than 20 new compounds could be detected in CM after the enzymatic treatment. The separation and spectral data of these products are given in Appendix E (Table E-3).

The composition of the products extracted from enzymatically treated CM changed with time, indicating that some of the detected products were only intermediates (Figure 9-4). After 15 min of the reaction, more than 13 new compounds were found in the MeOH extract of the treated meal. This number reduced to 5 (compounds 1, 8, 13, 15, 17) after 1 hour of processing, and eventually to two (compounds 1, 8) after 12 hour-long treatment. Peaks that are marked by the letter B in all of the chromatograms represent the artifacts originated from the buffer and the enzyme preparation (Figure 9-4a).

Figure 9-5a showed that the composition of the continuous phase was more complex than the composition of treated CM itself. However, the only new product found in the continuous phase after 15 minutes of enzymatic treatment that could be well-separated was compound 12. The compounds marked by letter A, were disregarded since they could have been present in the treated CM but were not detected because of the large artifact originated from the buffer (Figure 9-4a).

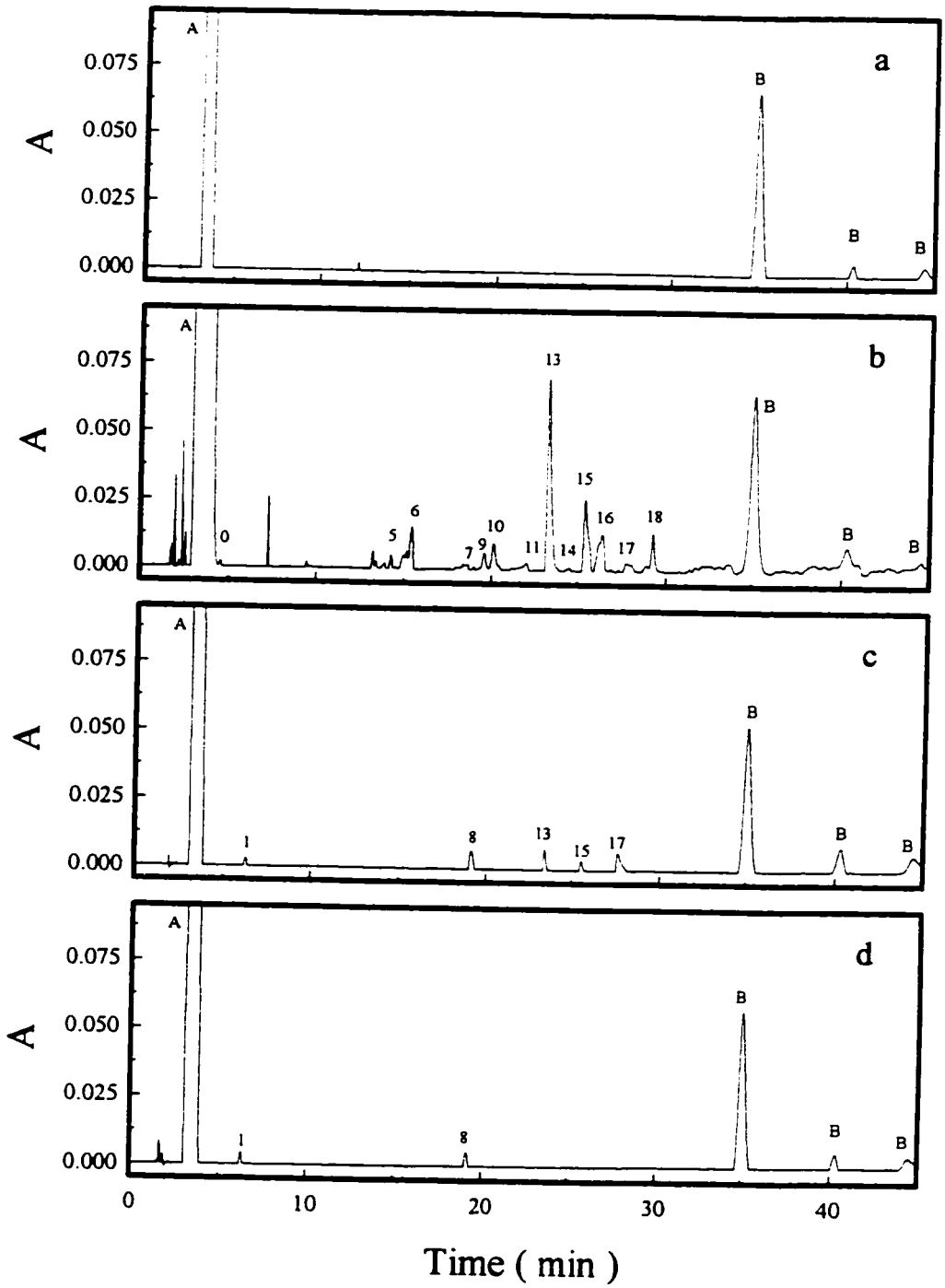


Figure 9-4. Chromatograms representing products found in canola meal after treatment with: (a) - deactivated enzyme for 3 hours; and the active enzyme for: (b) - 15 min; (c) 1 - hour; (d) -12 hours. Chromatograms obtained with MaxPlot method as described in Appendix D using initial CM composition as the reference state.

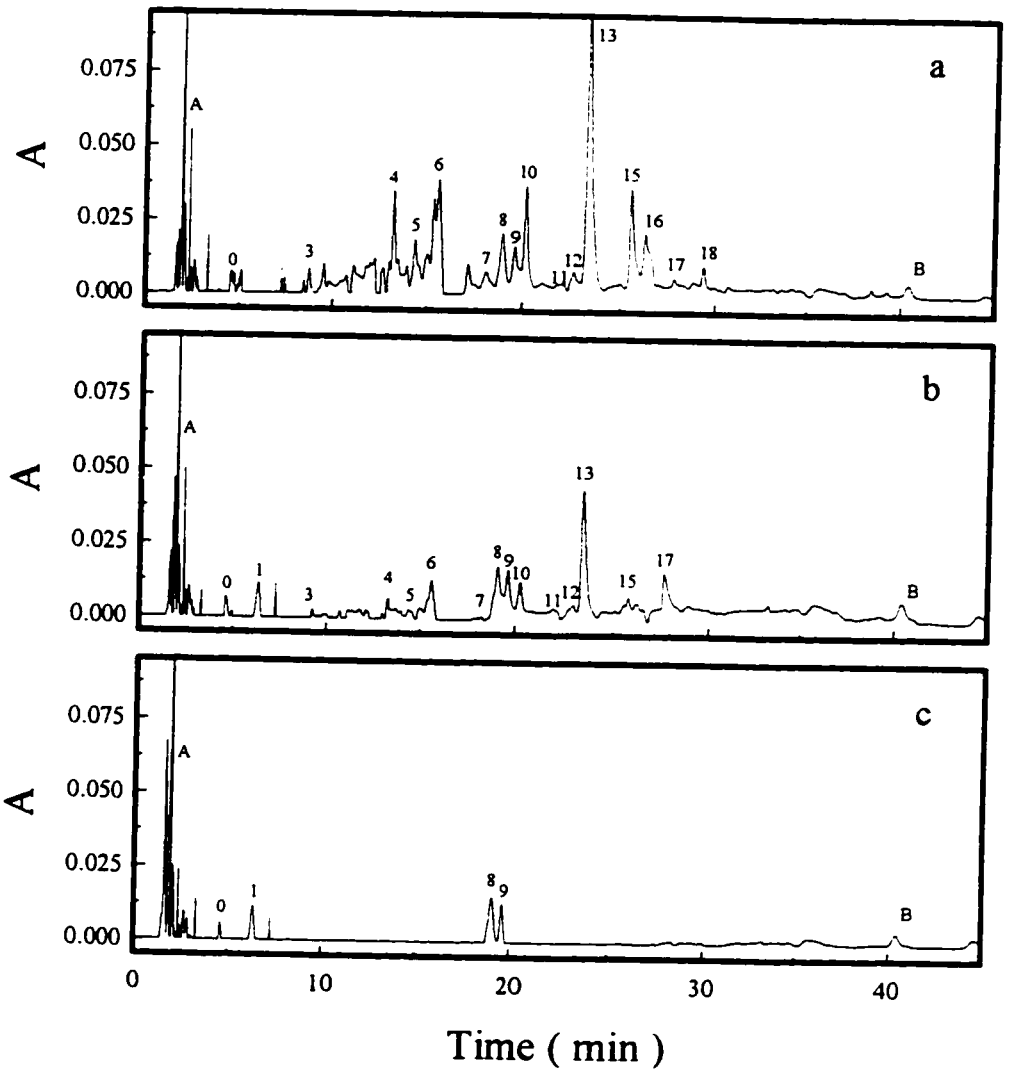


Figure 9-5. Chromatograms representing the composition of continuous phase from the enzymatic process consisted of a treatment with the active enzyme for (a) - 15 min, (b) - 1 hour, (c) - 12 hours. Chromatograms obtained applying MaxPlot method as describe in Appendix D using the composition of continuous phase from the system with the deactivated enzyme as the reference state.

Comparing Figure 9-4 and Figure 9-5 it could be concluded that the main product formed during the initial stages of the enzymatic process was compound 13. However, it underwent further transformations, and could not be detected in CM nor in the continuous phase after 12 h of processing.

The composition of the products fraction from the CM after 12 hours of the treatment and from the respective continuous phase were different, Figure 9-4d and Figure 9-5c, respectively. In addition to compounds 1 and 8, two products, compounds 0 and 9, were detected only in the continuous phase. This result showed that some of the products formed during the enzymatic treatment of CM were completely removed from the system upon separation of the meal and the continuous phase and, therefore, did not affect the quality of the meal.

To investigate whether some of the products formed during the enzymatic process were bound to the meal, the composition of the product fraction in the continuous phase from the system from which canola meal was removed prior to the addition of the active enzyme, was compared with the composition of this phase from the system when the meal was present during the enzymatic process. The results showed (Figure 9-6 initial part of the chromatogram) that the compositions of continuous phase in the system where CM was not present during the reaction was more complex than that of the continuous phase from the system in which CM was present (Figure 9-5). However, these new products were not separated very well by the HPLC method. Nevertheless, their absence in the samples representing the continuous phase from the actual enzymatic process showed that they were bound to the meal. This is confirmed when relative differences in peaks sizes, for example peaks 0 and 8 (Figure 9-5 and Figure 9-6), are considered. The hypothesis that some of the products are bound to the meal is also supported by the spectrophotometric data (Appendix E: Table E-3). They showed that the reddish color of the treated meal can be associated with the presence of compounds having absorbance maxima at 500-550 nm.

Identification of the detected products was based on the comparison of their UV-VIS spectra (Appendix E: Table E-3) with the spectra of the products of the enzymatic transformation of SA (Appendix E: Table E-1). Based on this comparison, compound 1 was identified as 2,6-dimethoxy-*p*-benzoquinone. Compound 2 also had similar light absorbing properties as *p*-quinone.

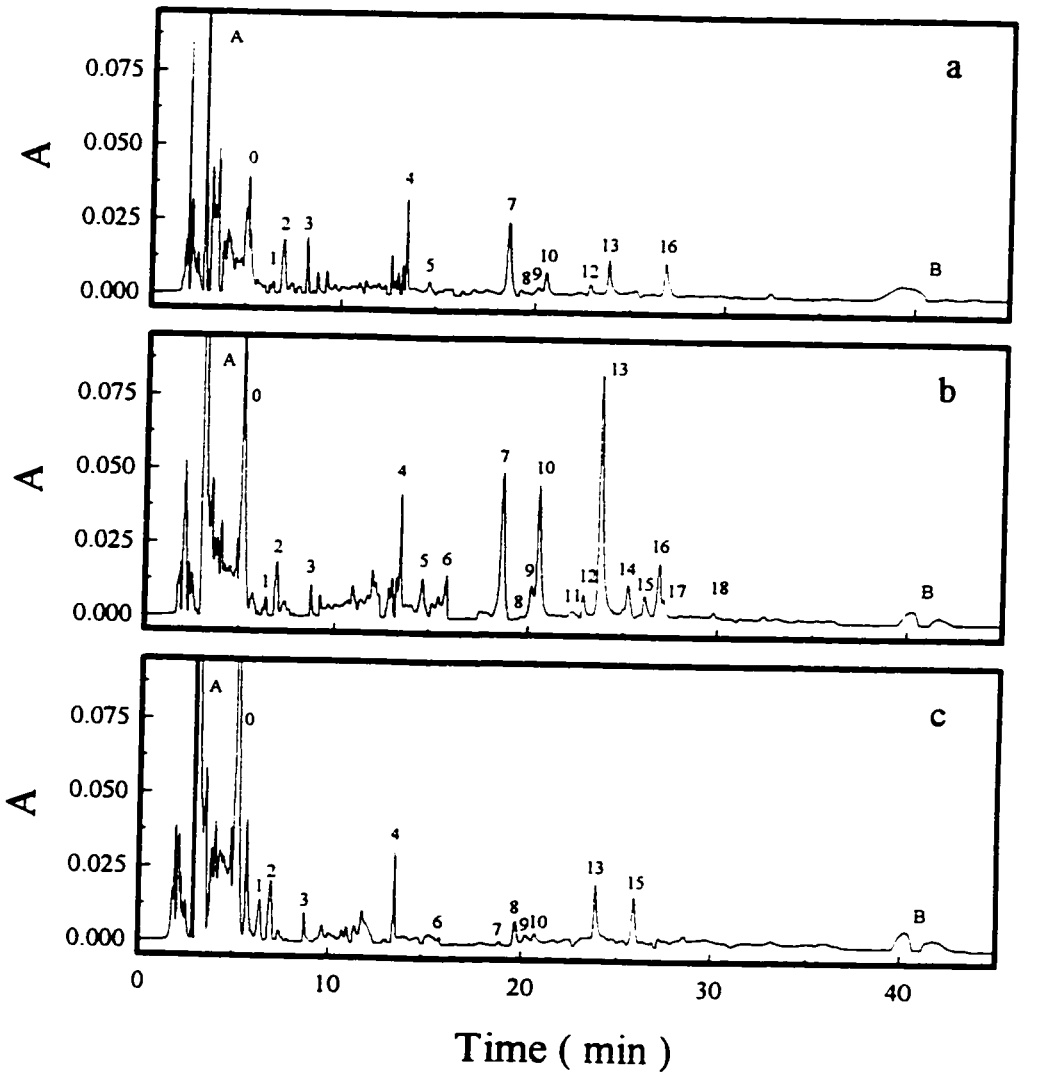


Figure 9-6. Chromatograms representing the composition of continuous-like phase from the enzymatic process without the presence of CM (a) 15 min, (b) 1 hour, (c) 12 hours. Chromatograms obtained with MaxPlot method as described in Appendix D using the initial composition as a reference state.

(Appendix E: Table E-3), but the identity of compound 1 was confirmed by a direct comparison of the retention time of the authentic 2,6-dimethoxy-*p*-benzoquinone with the retention times of compounds 1 and 2. 2,6-dimethoxy-*p*-benzoquinone was obtained by carrying out the enzymatic transformation of SA. The rest of the products were not identified. Considering that the enzymatically treated CM should be used as a raw material for food formulations, studies to identify the remaining products of the enzymatic transformation of CM phenolics and their impact on the meal quality should be performed. In addition, the insoluble-bound phenolic fraction should also be analyzed for new products because the chromatogram representing the composition of this fraction in the enzyme treated meal (Figure 9-5) suggested that some of the products from the enzymatic transformations of the soluble SAE and free SA were bound to the meal.

9.3 Determination of the SAE content

In this section, some aspects related to the methods of measuring the SAE content in the untreated and enzymatically treated meal will be presented.

9.3.1 Extraction of phenolics from canola meal

In order to determine which of the systems reported in the literature to extract phenolics from rapeseed meals would be the most suitable for the purpose of this work, the commercial canola meal was extracted using three different solvents. The results obtained (Table 9-4) showed

Table 9-4. Extraction of sinapic acid esters from commercially available canola meal using three different solvent systems.[a]

Extraction step	Extracted SAE [g/kg]			
	MeOH [b]	2%-HCl in MeOH [b]	Acetone-water [b]	MeOH [c]
I	18.21 (0.39)	19.96 (0.41)	20.72 (0.59)	18.63 (0.52)
II	2.05 (0.06)	1.02 (0.06)	1.99 (0.23)	-
III	0.69 (0.02)	0.26 (0.01)	0.67 (0.16)	-
Total	20.95 (0.47)	21.24 (0.48)	23.38 (0.98)	18.63 (0.52)

[a] SAE concentration measured using chemical spectrophotometric method
[b] boiling solvent, each extraction 20 minutes.
[c] 24 hour extraction at room temperature.

that a triplicate extraction with an acetone-water mixture was the most efficient, followed by extraction with acidified methanol. The acidified methanol extracted more SAEs from the meal than methanol alone, which is in agreement with the data reported on the extraction of tannins from CM [Blair and Richter, 1984]. Furthermore, the extraction with boiling MeOH for 20 minutes was more efficient than the 24 hour extraction at room temperature. When the total amounts of SAEs extracted by each of the considered solvents were compared at $p<0.05$ probability level, it was found that they were not significantly different. On the other hand, the results obtained after the first extraction step were significantly different at $p<0.05$. One explanation for a higher efficiency of extraction achieved after the first extraction step with the acetone-water system could be the extraction of some water soluble protein-phenolic complexes. Since a triplicate extraction of the meal with acetone-water and with acidified methanol did not produce significantly different results, for practical reasons the acidified methanol was used as an extracting solvent in this work.

9.3.2 Measurements of SAE content in enzymatically treated canola meal

The investigated process for the enzymatic decrease of phenolic content in canola meal was evaluated on the basis of the change in the concentration of all SAE before and after the treatment. Most of the measurements were done using two spectrophotometric analytical methods, direct (DS) and chemical (ChS). These methods have the same sensitivity as a HPLC method when used to analyze different types of untreated canola meals [Łącki and Duvnjak, 1996b], but they are nonspecific. The DS method measures phenolic content in the meal in terms of all SAE plus free SA, whereas the ChS method measures all SAE. The non specificity of these methods was not considered a disadvantage in this work because the project was focused on the elimination of all of the phenolics present in the meal. However, it was noticed during the course of this study, that an interference originating from the enzymatic treatment affected the measurements of the SAE concentration using these methods (Appendix C).

To evaluate the extent of the observed interference, the methanol extracts of untreated meal and that which was treated for 1 and 16 hours with the active enzyme were analyzed for SAEs using both spectrophotometric methods and the HPLC method. To rule out the possibility that the interference was related to the origin of the meal, 6 meals from different canola cultivars were also

analyzed in addition to two kinds of commercial meal. The HPLC quantitation of all SAE was based on the SIN standard curve as explained in Appendix C. The free SA was measured according to the standard curve prepared with an authentic sample. Separate quantitation of the free SA and of all the SAEs allowed a comparison of the results obtained using HPLC with those obtained by the spectrophotometric techniques.

The results obtained by the three analytical techniques are given in Table 9-5. Their comparison showed that the total concentration of SAE and/or SAE-SA in the untreated meals was practically the same regardless of the technique used. The results obtained using the HPLC and the ChS methods showed that in each type of meal, the total SAE concentrations measured using either of these methods did not differ significantly when compared using a two sided *t* test at 0.05 significance level. Lower concentrations of SIN determined by the ChS method compared to the HPLC method could be explained by the efficiency of the precipitation step involved in the Reinecke salt method for separating SIN from the methanol extract. Austin and Wolff [1968] reported that this salt was only 90.0% efficient in the complexation of sinapine, which is comparable to the 86.0% observed in this work. According to the results from the HPLC analysis, SIN represents on average 66.3% of all SAE, whereas the free SA was found to be in the range of 9.0% to 15.0% of the total phenolics content expressed as the SA equivalent. Both of these results are in agreement with the reported data [Mueller *et al.*, 1978a; Krygier *et al.*, 1982b; Bouchereau *et al.*, 1991].

Comparing the HPLC and DS methods, it was noted that both of them produced similar results. The DS method measured the phenolic content in terms of all SAE plus free SA and, therefore, the concentration obtained by this method should be comparable to the combined concentration of these compounds determined by HPLC.

In general, the results regarding SAE concentrations in the untreated meals showed that any of the three methods examined can be used for the determination of the phenolic content in the rapeseed meals. Since the spectrophotometric methods are more facile than the HPLC separation, they could be recommended for SAE screening purposes. Furthermore, the combination of the results obtained by both spectrophotometric methods would result in the estimation of the

Table 9-5. The concentrations of SAE, SIN and SA in the untreated and treated* meals measured by different analytical methods.

Stage	Meal	Concentration (g/kg) when measured by:					
		DS	ChS		HPLC		
		SAE + SA	SAE	SIN	SAE	SA	SIN
Initial	Ceres	36.46 (0.74)	31.21 (1.11)	16.50 (0.86)	32.81 (0.78)	5.48 (0.40)	19.72 (0.34)
	Profit	34.02 (0.22)	28.37 (1.24)	16.99 (1.30)	27.13 (0.80)	3.88 (0.22)	19.81 (0.97)
	Falcon	37.59 (0.10)	31.67 (1.78)	16.85 (0.90)	28.61 (1.75)	4.22 (0.68)	17.54 (0.87)
	K32-87-16	32.57 (0.42)	29.03 (0.64)	17.83 (0.22)	28.14 (0.59)	5.10 (0.20)	18.06 (0.21)
	Cyclone	42.72 (1.31)	35.06 (0.35)	20.18 (1.22)	38.03 (1.32)	5.24 (0.28)	27.82 (1.44)
	Eldorado	33.19 (0.52)	29.31 (1.45)	14.66 (0.15)	29.68 (0.62)	4.82 (0.12)	18.10 (0.21)
	CM (I)	26.02 (0.70)	21.24 (0.48)	12.40 (0.18)	22.62 (0.94)	2.24 (0.06)	15.03 (0.39)
	CM (II)	28.28 (0.10)	23.52 (1.32)	13.59 (0.55)	20.07 (0.08)	2.98 (0.3)	14.31 (0.29)
	Ceres	9.89 (0.37)	6.41 (0.15)	nd	1.92 (0.04)	0.28 (0.02)	1.04 (0.14)
1 h	Profit	10.89 (0.11)	6.80 (0.81)	nd	1.96 (0.02)	0.38 (0.02)	1.44 (0.17)
	Falcon	12.27 (0.13)	7.26 (0.10)	nd	2.20 (0.06)	0.32 (0.10)	1.08 (0.15)
	K32-87-16	8.84 (0.21)	5.30 (0.42)	nd	1.40 (0.05)	0.24 (0.02)	1.07 (0.21)
	Cyclone	7.58 (0.79)	5.68 (0.53)	nd	2.46 (0.04)	0.30 (0.06)	1.56 (0.08)
	Eldorado	10.73 (0.05)	6.68 (0.55)	nd	2.28 (0.06)	0.36 (0.04)	0.61 (0.21)
	CM (I)	7.58 (0.30)	4.60 (0.51)	2.54 (0.61)	1.55 (0.05)	0.14 (0.01)	1.01 (0.04)
	CM (II)	7.73 (0.78)	4.88 (0.54)	nd	1.12 (0.07)	0.16 (0.06)	0.79 (0.03)
16 h	Ceres	4.31 (0.13)	1.92 (0.03)	nd	0.52 (0.02)	0.06 (0.01)	0.20 (0.01)
	Profit	4.20 (0.06)	2.29 (0.04)	nd	0.44 (0.02)	0.04 (0.01)	0.18 (0.01)
	Falcon	4.57 (0.08)	2.67 (0.16)	nd	0.50 (0.02)	0.06 (0.01)	0.30 (0.01)
	K32-87-16	3.40 (0.10)	1.73 (0.08)	nd	0.48 (0.04)	0.02 (0.01)	0.18 (0.01)
	Cyclone	4.14 (0.39)	2.76 (0.77)	nd	0.54 (0.12)	0.24 (0.07)	0.56 (0.05)
	Eldorado	3.43 (0.01)	2.29 (0.10)	nd	0.34 (0.02)	0.16 (0.01)	0.22 (0.01)
	CM (I)	4.69 (0.05)	2.00 (0.02)	1.23 (0.08)	0.43 (0.06)	0.05 (0.01)	0.38 (0.06)
	CM (II)	4.11 (0.50)	2.32 (0.32)	nd	0.38 (0.02)	0.07 (0.01)	0.24 (0.02)

* meal incubated in the presence of the citrate-phosphate buffer, pH 4.5, and oxidase enzyme at 50°C, with continuous mixing.
nd - not determined
values in brackets represent standard deviationss

concentration of SAE and the free SA. When the extent of the enzymatic treatment was evaluated using the three techniques considered, significant discrepancies were observed (Table 9-5). The HPLC results showed that a 16 hour-long enzymatic treatment of 8 different canola meals resulted in at least 98.0% reduction of the SAE content. In contrast, the spectrophotometric methods underestimated this result by 7.0%. The underestimation was even higher when a 1 hour-long treatment was considered (Table 9-5). In this case, the results obtained with the spectrophotometric methods showed a 78.0% decrease in the SAE content of the meal

compared to a 93.0% decrease measured using the HPLC method. The higher values of the residual SAEs measured using the spectrophotometric methods were a result of the interference that had been caused by products of the enzymatic reaction that were present in the methanol extract from the treated meals (Appendix C).

The result regarding enzymatically treated meals (Table 9-5) showed that the spectrophotometric methods can not be directly used to obtain representative results regarding the extent of phenolic content decrease in enzymatically treated CM. On the other hand, data from Table 9-5 can be used to find the sensitivity limits for both spectrophotometric techniques when used to measure SAE content after the enzymatic process. Plotting the results obtained with each of the spectrophotometric methods against these obtained with HPLC, and performing least-square analysis to the following correlation between the results obtained by these methods were found:

$$C_{ChS} = 1.947\, C_{HPLC} + 1.466 \qquad (R^2 = 0.863) \tag{9.1}$$

$$C_{DS} = 3.064\, C_{HPLC} + 2.674 \qquad (R^2 = 0.836) \tag{9.2}$$

where: C_{ChS}, C_{DS}, and C_{HPLC} are the SAE concentrations (g/kg) measured with the ChS, DS and HPLC methods, respectively.

The fact that the slopes of these relationships are larger than one indicates that the overestimation of the SAE concentration observed with spectrophotometric methods could not be caused by a constant interference, but had to be proportional to the amount of the SAE enzymatically transformed. Furthermore, the intercepts of Eq.(9.1) and Eq.(9.2) should be considered as a sensitivity limit for the spectrophotometric methods in determining the phenolics content in the enzymatically treated meal.

Considering the above, some of the results presented later in the text which were obtained with the spectrophotometric methods, mainly ChS, were recalculated taking into consideration the sensitivity limits for these methods, and the fact that the interference from the products of the enzymatic treatment was proportional to the amount of SAE converted by the enzyme, as described in Appendix C.

9.4 Search for the model variables

To model the enzymatic process for decreasing the phenolic content in CM, it was necessary to know the kinds of physical phenomena involved, and which of the system variables affects the process the most. In order to gain this information, experiments focusing on the effects of the system variables, such as enzyme and meal concentrations, time of the process, temperature, pH, particle size, and mixing on the extent of SAE content reduction were performed.

9.4.1 Dynamics of SAE content reduction

Three series of experiments were performed to study the dynamics of the enzymatic reduction of the phenolics content in CM during the first hour of the process at different meal and enzyme concentrations. In each series, the meal concentration varied from 6.6 to 20.0% (w/v). In the first series, the enzyme concentration in the continuous phase, which from now on will also be called the enzyme working concentration, was 4.0 nkat/mL. In the second series, the enzyme concentration per unit mass of the dispersed phase was 20.0 nkat/g. In the third series, treatment with the deactivated enzyme was considered. The results obtained in the enzyme-free systems, which served as the control, showed (Figure 9-7) that the buffer extraction alone resulted in a 30.0% and 55.0% decrease in SAE content for systems with meal concentrations of 20.0% and 6.6%, respectively. It was also noted that the extraction of SAE was completed within the first 15 minutes of the treatment. Addition of the active enzyme to the continuous phase resulted in a substantial decrease in SAE concentration (Figure 9-7). At least an additional 30.0% decrease was achieved in the systems where the enzyme concentration was kept constant in the continuous phase. The rates of the enzymatic process were the highest at the beginning of the treatment, and started leveling off as the concentration of the SAE in the meal decreased. This type of response suggested that either the concentration of the enzyme was decreasing or that the extraction of SAE from the meal matrix became the rate limiting step. The results obtained for the meal concentration of 20.0% showed, however, that the enzymatic reaction was occurring at an almost constant rate during the whole 60 minutes of the treatment. Thus, it could be concluded that as the concentration of SAE in the meal decreased, the extraction became the rate limiting step of the overall process.

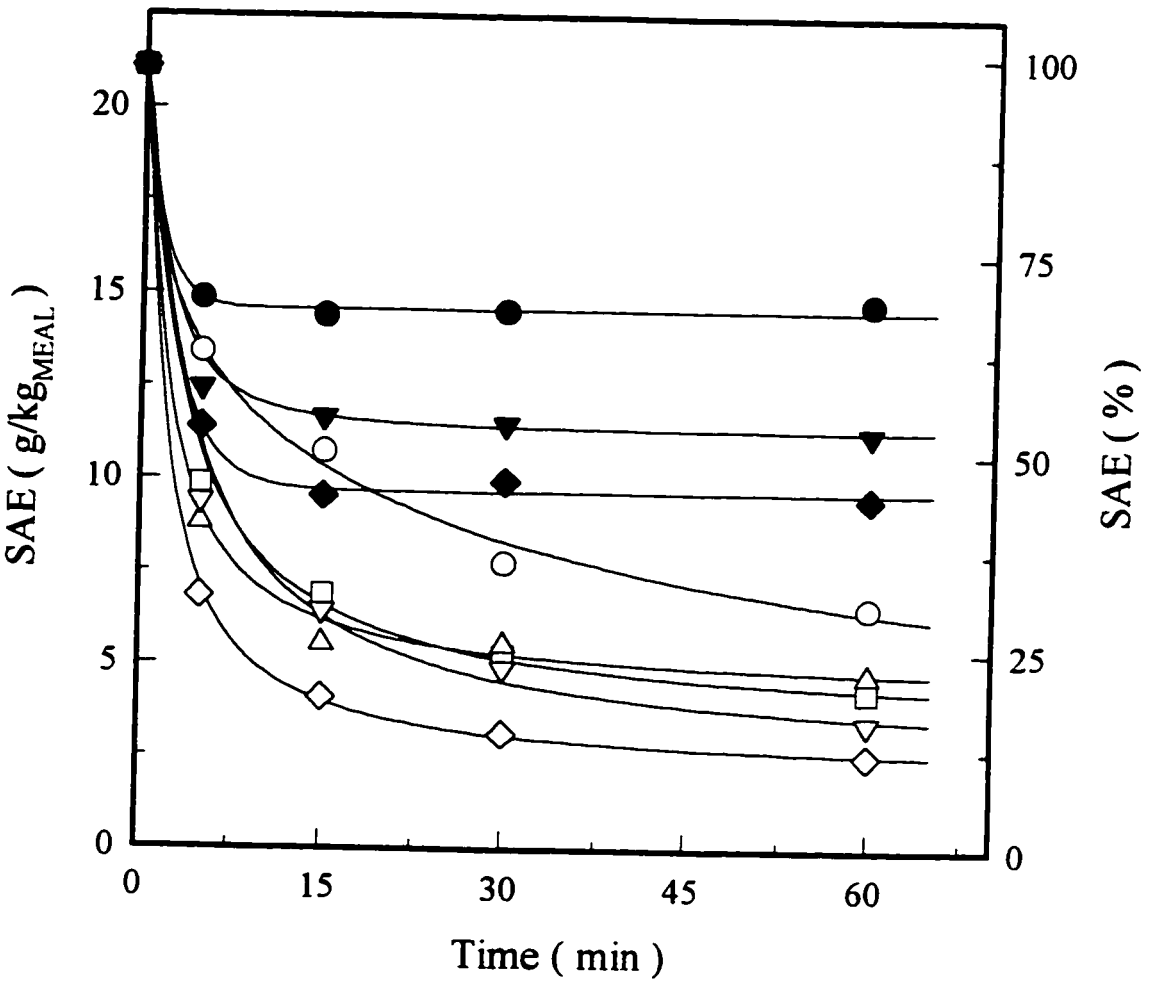


Figure 9-7. Dynamics of SAE content reduction for different ratio of continuous phase to meal (v/w). Ratio 5.0: (○) 4.0 nkat/mL; (●) 0.0 nkat/mL. Ratio 10.0: (▽) 4 nkat/mL; (□) 2 nkat/mL; (▼) 0.0 nkat/mL. Ratio 15: (◊) 4.0 nkat/mL; (Δ) 1.33 nkat/mL; (◆) 0.0 nkat/mL. Conditions: 30°C, pH 4.5.

When the enzyme concentration per unit mass of CM was kept constant, *i.e.*, a decrease in the working enzyme concentration with a decrease in the meal concentration, it was found (Figure 9-7) that the extent of SAE decrease was the same in the systems where the meal concentrations were 10.0% and 6.6% (w/v). This result showed that the increase in the enzyme concentration from 1.33 nkat/mL to 2.0 nkat/mL compensated for the decrease in the volume of the continuous phase by 33.0%. On the other hand, a decrease from 20.0% to 10.0% in the meal concentration accompanied by a two fold decrease in the enzyme concentration caused an additional decrease in the SAE content by at least 10.0%.

Considering the above, it appeared that there may be an optimum concentration for the meal suspension, at which the gain due to the addition of the enzyme would be the greatest. To examine this hypothesis, the effect of meal and enzyme concentrations on the rate of SAE content decrease were studied in more detail.

9.4.2 Effect of meal concentration

To investigate the effect of the meal concentration on the rate of SAE content decrease, series of experiments were performed using systems with constant enzyme concentration per milliliter of the continuous phase (liquid: buffer plus enzyme) or per gram of the dispersed phase (meal). In these tests, the concentration of CM varied from 1.0% to 49.0% (w/v). For the enzyme-free systems with meal concentrations below 20.0% (w/v), a decrease in SAE content was almost proportional to the decrease in the meal concentration (Figure 9-8). At higher meal concentrations, the proportionality disappeared and the extent of SAE extraction became independent of the meal concentration. These results showed that the extraction process was governed by an equilibrium between SAE concentrations in the continuous and dispersed phases.

The results obtained in the experiments with the active enzyme showed that the residual SAE concentration in the meal was always lower in the systems with the higher enzyme concentration, regardless of the meal concentration (Figure 9-8). The lack of significant differences in the systems with a meal concentration of 49.0% (w/v) was caused by a poor distribution of the enzyme throughout the system. At this meal concentration there was no free continuous phase owing to the imbibery properties of canola meal.

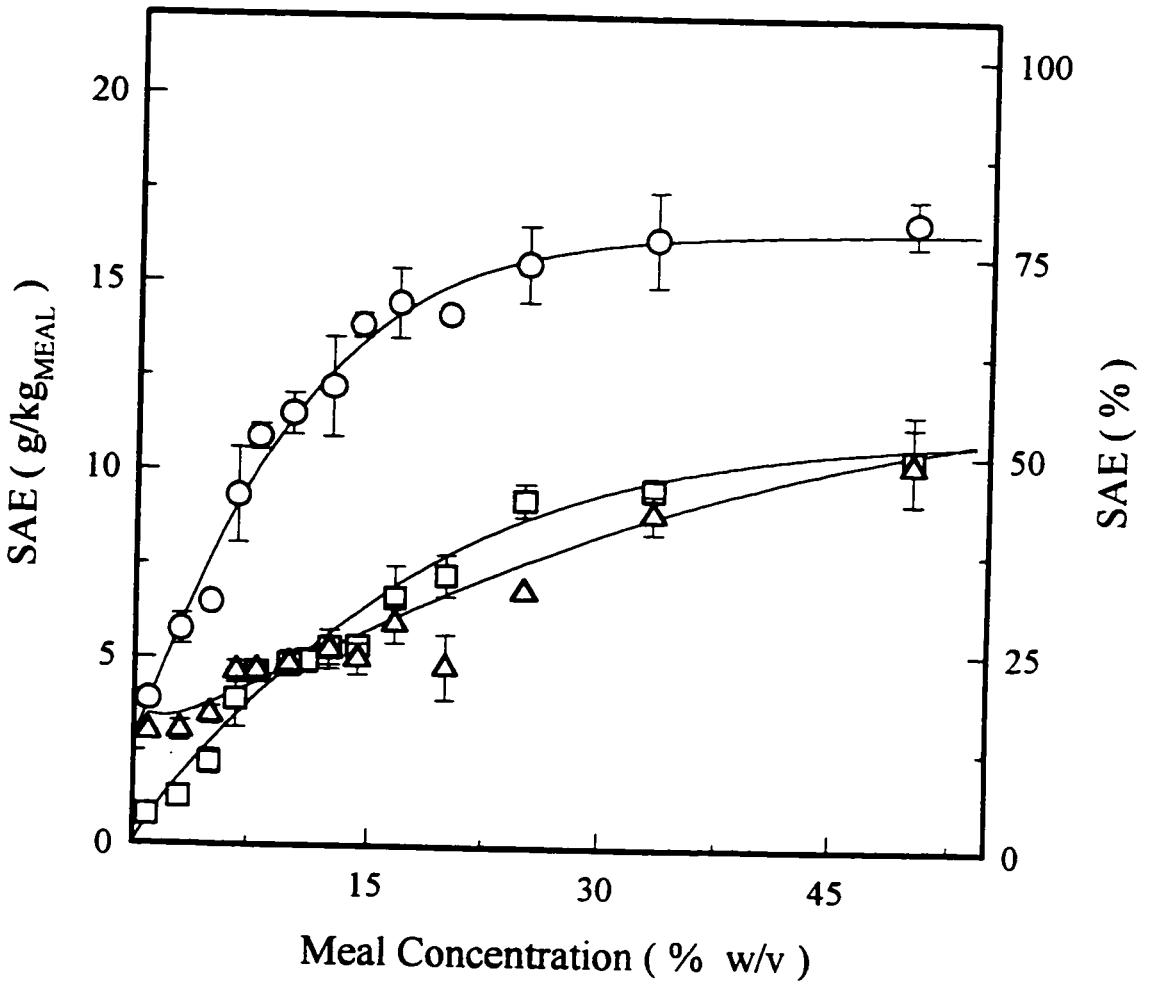


Figure 9-8. Effect of meal concentration on the decrease of SAE content in canola meal using systems with: (O) deactivated enzyme; (□) constant enzyme concentration in the continuous phase, 1.0 nkat/mL; (Δ) constant enzyme concentration per gram of dispersed phase, 10.0 nkat/g. Conditions: temperature 30°C, pH 4.5, reaction time 30.0 min.

The intersection pattern for the data representing systems with constant enzyme concentrations per unit volume of the continuous and per unit mass of the dispersed phases (Figure 9-8) can be explained through a decline in the respective enzymatic reaction rates caused by changes in the working enzyme concentration. For meal concentrations higher than the one at which the intersection was observed, the enzyme concentration in the continuous phase was always higher in the systems in which the amount of the enzyme per gram of the meal was kept constant. On the other hand, for the meal concentrations lower than the one at which the intercept occurred, the working enzyme concentration was always higher in the system with the constant concentration of the enzyme in the continuous phase, *i.e.*, in which the amount of the enzyme in the system increased with the decrease in the meal concentration. The decrease in SAE concentration in the continuous phase due to the decrease in the meal concentration does not have to be included in the above discussion since it affected both systems in the same way. The meal concentration at which the intersection pattern should appear can be calculated from the ratio of the enzyme concentration expressed per mL of the continuous phase to the concentration per unit mass of the meal.

9.4.3 Effect of enzyme concentration

As shown in the previous section, the enzyme concentration had a considerable effect on the outcome of the process and, therefore, it was investigated in more detail. It was especially of interest to determine whether the system with a fixed concentration of CM could be saturated with the enzyme. In these tests, the enzyme concentration varied from 0.0 nkat/g to 190.0 nkat/g with a constant meal concentration of 20.0% (w/v). As shown in Figure 9-9 the rate of decrease in the SAE content was directly proportional to the increase in the enzyme concentration for concentrations up to 7.0 nkat/g. Above this concentration, the proportionality started to disappear and, eventually, the increase in enzyme concentration from 40.0 to 190.0 nkat/g only resulted in an additional 5.0% decrease in the SAE content. This result was confirmed by observing the development of a red colour in the reacting system during the enzymatic process. For the enzyme concentrations above 12.0 nkat/mL, the colour appeared within 10 minutes after enzyme addition without any noticeable difference with respect to the increase in enzyme concentration. The appearance of the same colour during the enzymatic conversion of SA in the model system was

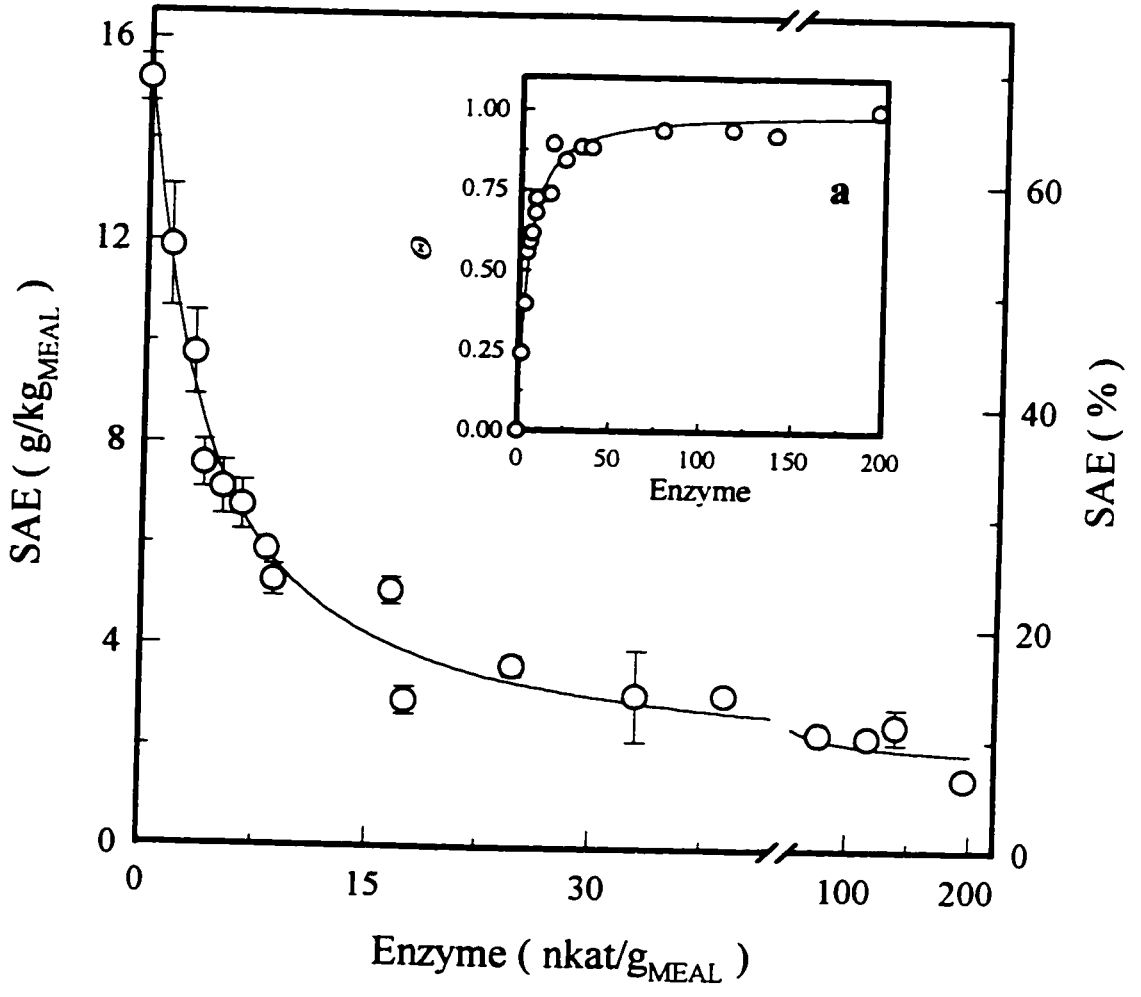


Figure 9-9. Effect of enzyme concentration on the decrease of the SAE content in canola meal and on the degree of saturation of the system with enzyme, Θ (Figure a). Conditions: temperature 50°C, pH 5.0, reaction time 1 hour, meal concentration 20.0% (w/v).

associated with the enzymatic transformation of DAD (section 8.2.6). This result indicated that the enzymatic transformations of the SAE and the free SA extracted from the meal were accompanied by the enzymatic reactions involving the products of these transformations.

The results presented in Figure 9-9 showed that the rate of the SAE content reduction can not be described by a typical first order reaction, with respect to the enzyme concentration, as was assumed in the models describing the enzymatic transformation of SA and SIN (Sections 8.3 and 8.4). In order to use these models in the description of the enzymatic process, it was necessary to take into consideration the saturation of the system with the enzyme. On the basis of the data presented in Figure 9-9, it was possible to propose a formula that correlates the degree of saturation of the system with the enzyme and the enzyme concentration expressed per unit mass of the treated meal. The degree of saturation of the system with the enzyme, Θ, was defined as the ratio of the difference between the SAE residual contents obtained at a given enzyme concentration and that in the enzyme-free system to the difference between the enzyme saturate and enzyme free systems. Such a definition of Θ implies that the degree of saturation can vary from 0.0 to 1.0. The values of Θ obtained by recalculating the data presented in Figure 9-9 are shown in Figure 9-9a. They were described using Eq.(9.3).

$$\Theta = \frac{1}{1 + \left(\frac{1}{p1} \frac{e_0 v_{enz}}{M_{CM}} \right)^{p3}} \tag{9.3}$$

where: Θ is the degree of saturation, $p1$ and $p3$ are the model parameters, M_{CM} mass of CM (g), e_0 concentration of the enzyme in the stock solution (nkat/mL), v_{enz} - volume of the enzyme added to CM (mL).

The parameters in Eq.(9.3) were estimated using a nonlinear-least squares method and were found to be: $p1 = 4.05$ (nkat/g) and $p3 = 1.01$.

9.4.4 Effect of pH

The effect of the pH of the buffer used to provide the continuous phase during the enzymatic treatment of CM on the extent of the decrease in the SAE content was also considered.

In the model system, the changes in the pH of the reacting solution had a strong effect on the enzyme activity. A drop in pH from its optimum value of 4.2 to 2.8 resulted in a 40.0% decrease in activity (Figure 8-2). In the case of CM (Figure 9-10), the same change in pH of the buffer resulted in only a 1.5% difference in the residual amounts of SAE. In both cases the enzymatic treatment produced a meal with about one third of the initial SAE concentration. In addition, the results (Figure 9-10) showed that the enzymatic reaction also took place when the buffer with pH 7.5 was used, although the decrease in the meal phenolic content was only slightly greater than the one caused by the buffer extraction alone. At the same pH level, the enzyme did not show any activity in the model system (Figure 8-2).

These results suggested that the pH of the CM system was not the same as that of the buffer. In fact, due to the buffering capacity of CM, the pH of the process depended on both the pH of the buffer and the CM concentration (Figure 9-11). At 50°C and at a 20.0% meal suspension, the pH of the system oscillated within a range of 4.5 to 5.5, even though the buffer pH was between 3.0 to 6.5, respectively. The same buffering effect of CM on the pH of the process was noticed at 30°C when an enzymatic decrease of the tannin content in CM was investigated [Larusso *et al.*, 1996].

The buffering capacity of CM is most likely related to the extraction of proteins from the meal into the continuous phase. This is shown in Figure 9-12, where the time changes in the pH of the system and in the amount of protein extracted from the meal are compared. An almost direct proportionality is well documented. Moreover, Figure 9-12 also indicates that the changes in the system pH were so fast, compared to the time-scale of the investigated enzymatic process, that it can be assumed that the enzymatic treatment was taking place at a constant pH.

The postulate that the pH of the continuous phase depends on the CM proteins was confirmed when the changes in the concentration of extracted proteins, as a function of the system pH and of the meal concentration, were considered (Figure 9-13). The proportionality between the concentration of the extracted proteins and the meal concentration, showed that the stronger buffering capacity observed in the systems with higher meal concentrations (Figure 9-11) were associated with higher concentrations of the extracted proteins.

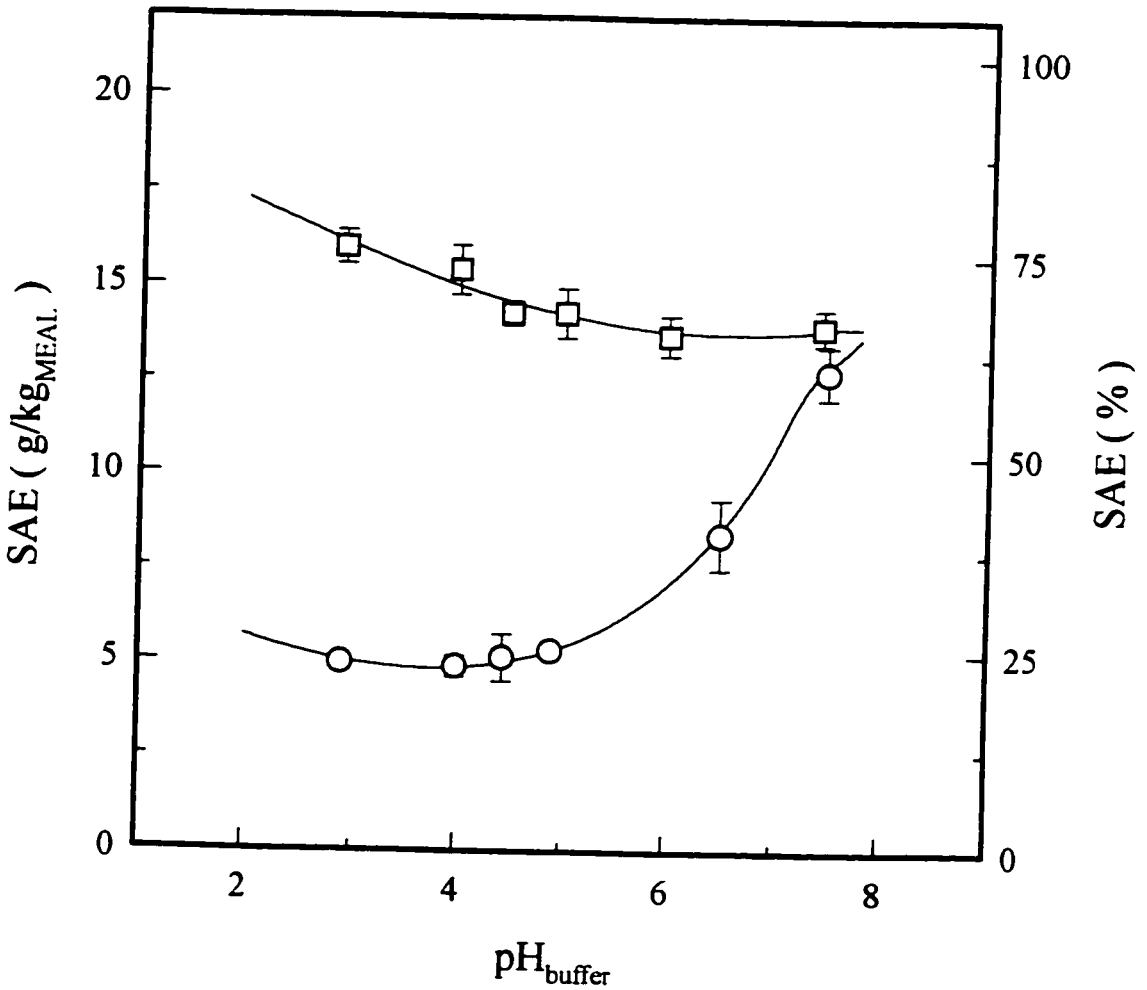


Figure 9-10. Effect of the pH of the buffer on the decrease of SAE content in CM at 50°C. Meal concentration 20.0% (w/v) using enzyme concentration 20.0 nkat/g: active enzyme (O); deactivated enzyme (□).

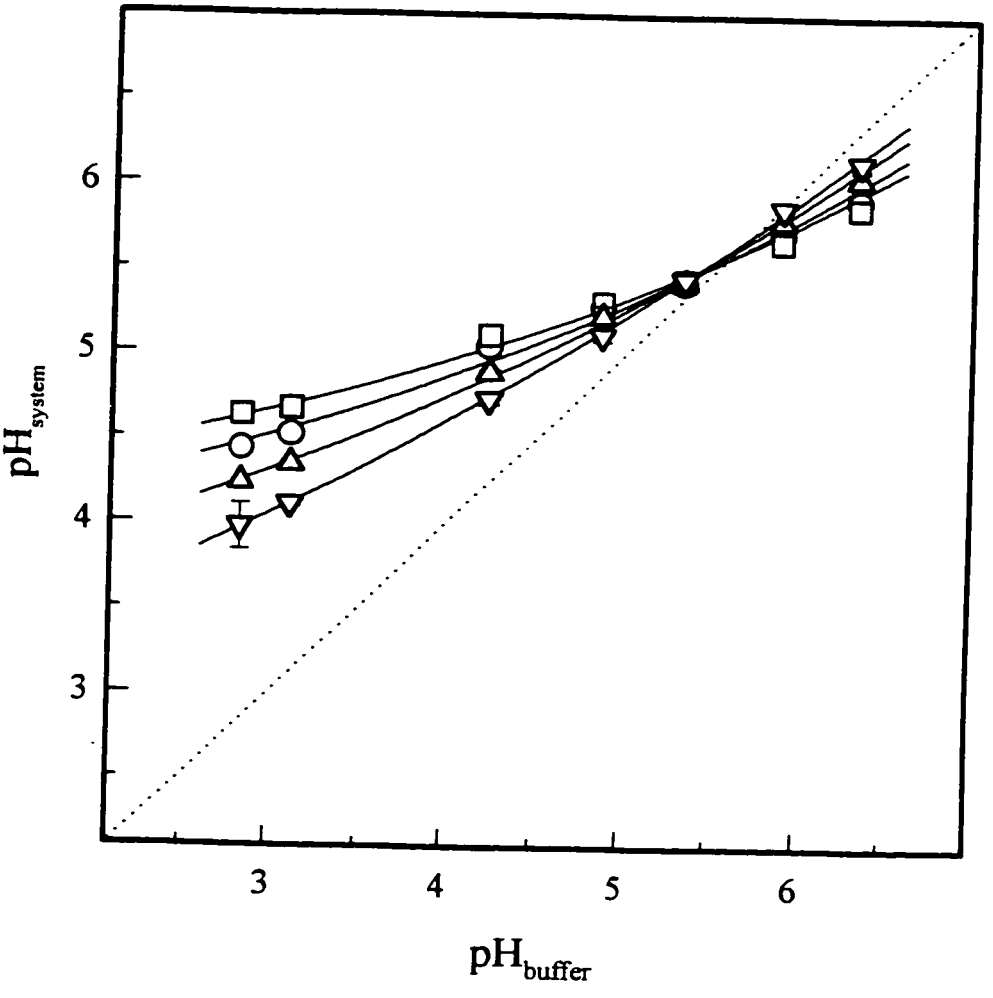


Figure 9-11. Effect of the pH of the buffer and the slurry concentration on the pH of the system at 50°C. Meal concentration: (∇) 5.0%; (Δ) 10.0%; (○) 15.0%; (□) 20.0%.

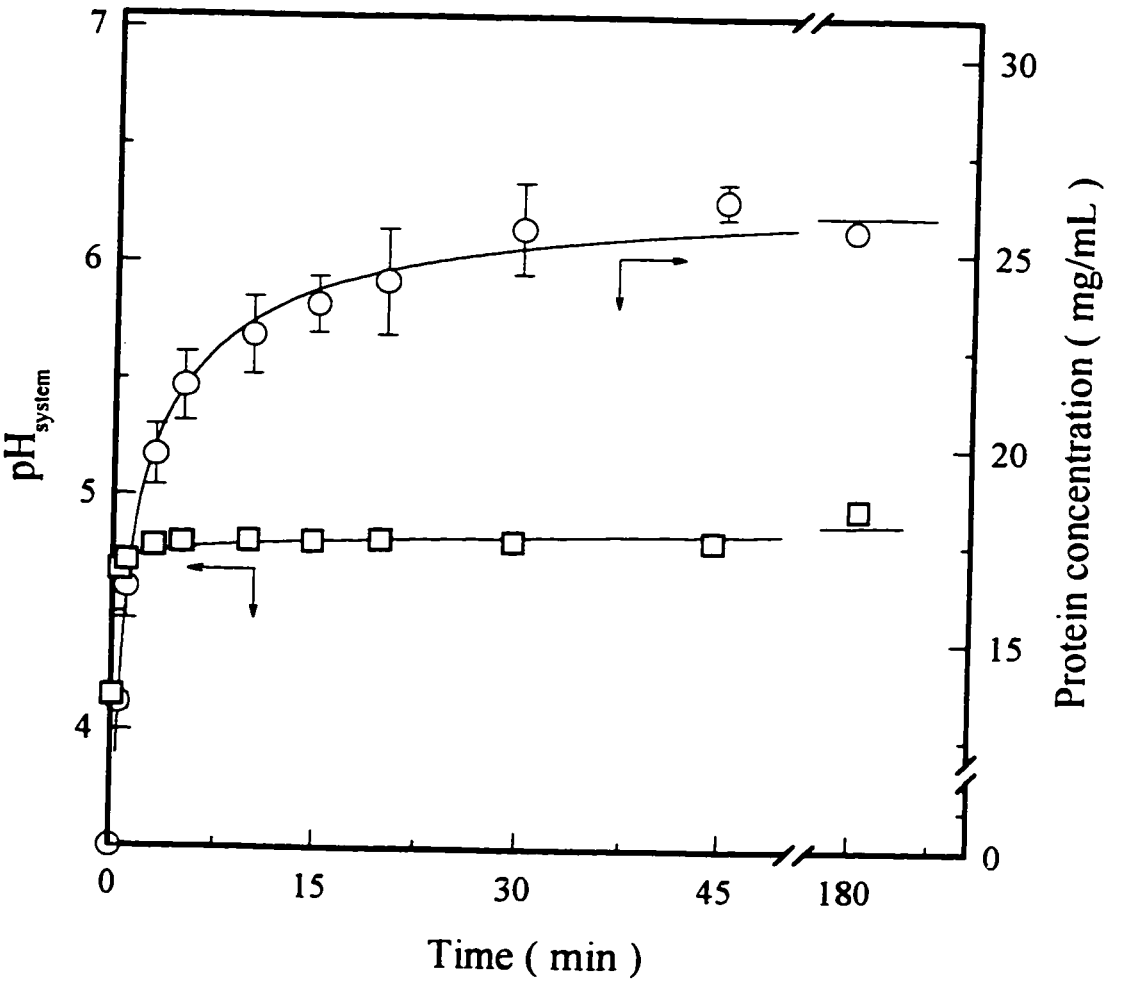


Figure 9-12. Time changes in the pH of the system and the amount of the extracted proteins at 50°C with meal concentration of 20.0%. Proteins - (○); pH - (□).

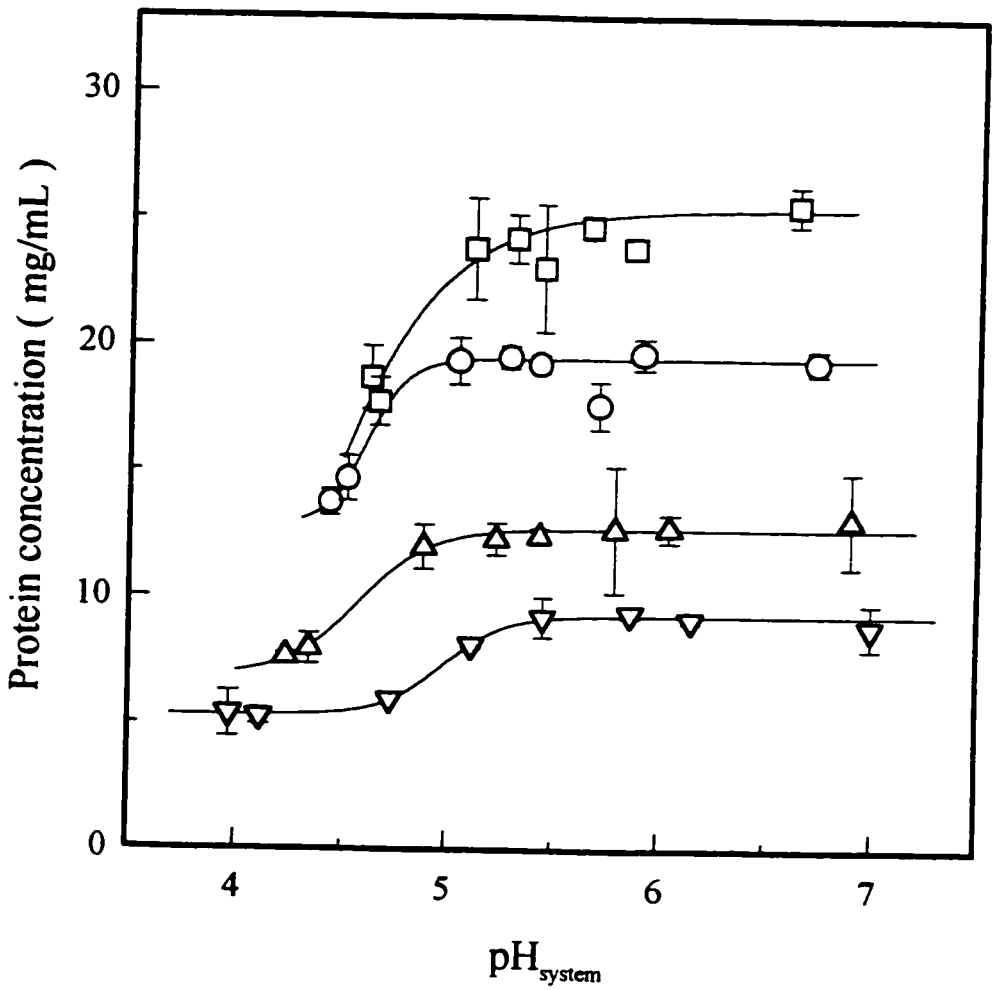


Figure 9-13. Effect of the system pH and the meal concentration on the amount of proteins extracted from CM at 50°C. Meal concentration: (▽) 5.0%; (△) 10.0%; (○) 15.0%; (□) 20.0%.

Considering the buffering capacity of CM and the amount of proteins extracted at the resulting pH levels (on average only 25.0% of the total protein content in CM), the effect of the system pH on the decrease in SAE content in CM was not investigated. Consequently, this effect was not included in the formulated model. Yet, if required, it could be taken into consideration in using the relations showed in Figure 9-11, and through the model describing the effect of pH on enzyme activity using Eq.(8-17).

9.4.5 Effect of temperature

Characterization of the enzyme in the model system showed that the enzyme possesses thermophilic properties, which leads to a well-defined optimum temperature of the reaction (Figure 8-4). Therefore, it was expected that temperature would similarly affect the rate of SAE decrease in CM. To investigate this, tests were carried out at temperatures ranging from 20°C to 70°C. The results obtained after 1 hour of treatment at two enzyme concentrations, 1.0 and 20.0 nkat/g, are presented in Figure 9-14. The enzyme concentration of 1.0 nkat/g was used to assure that the rate of the overall process measured after 30 minutes of the treatment was still reaction dependent. In this respect, a low enzyme concentration guaranteed that the results obtained represented the initial rate of the process, and did not include the limitations related to the extraction of SAE from the meal at low levels of phenolics.

The highest rate of SAE decrease was observed at 50°C, regardless of the enzyme concentration. This optimum temperature was similar to the one obtained with sinapine in the model enzymatic system. However, the effect of temperature on the rate of SAE decrease in CM (Figure 9-14) was not as profound as the one observed in the model system (Figure 8-4). For instance, changes in temperature from 30°C to 50°C in the model and CM systems, showed a difference in activity of 90.0% and 71.0%, respectively. Similarly, a change of 97.0% and 79.0% in the activity in these two system was observed when temperature was increased from 50°C to 70°C. Considering that the process temperature also has some effects on the extraction of SAE from CM (Figure 9-14), it can be seen that the effect of temperature on the initial rates of the process in the active enzyme system was even weaker. For temperatures above 50°C, the rate was virtually the same when only the pure reaction was considered, *i.e.*, subtracting the buffer results.

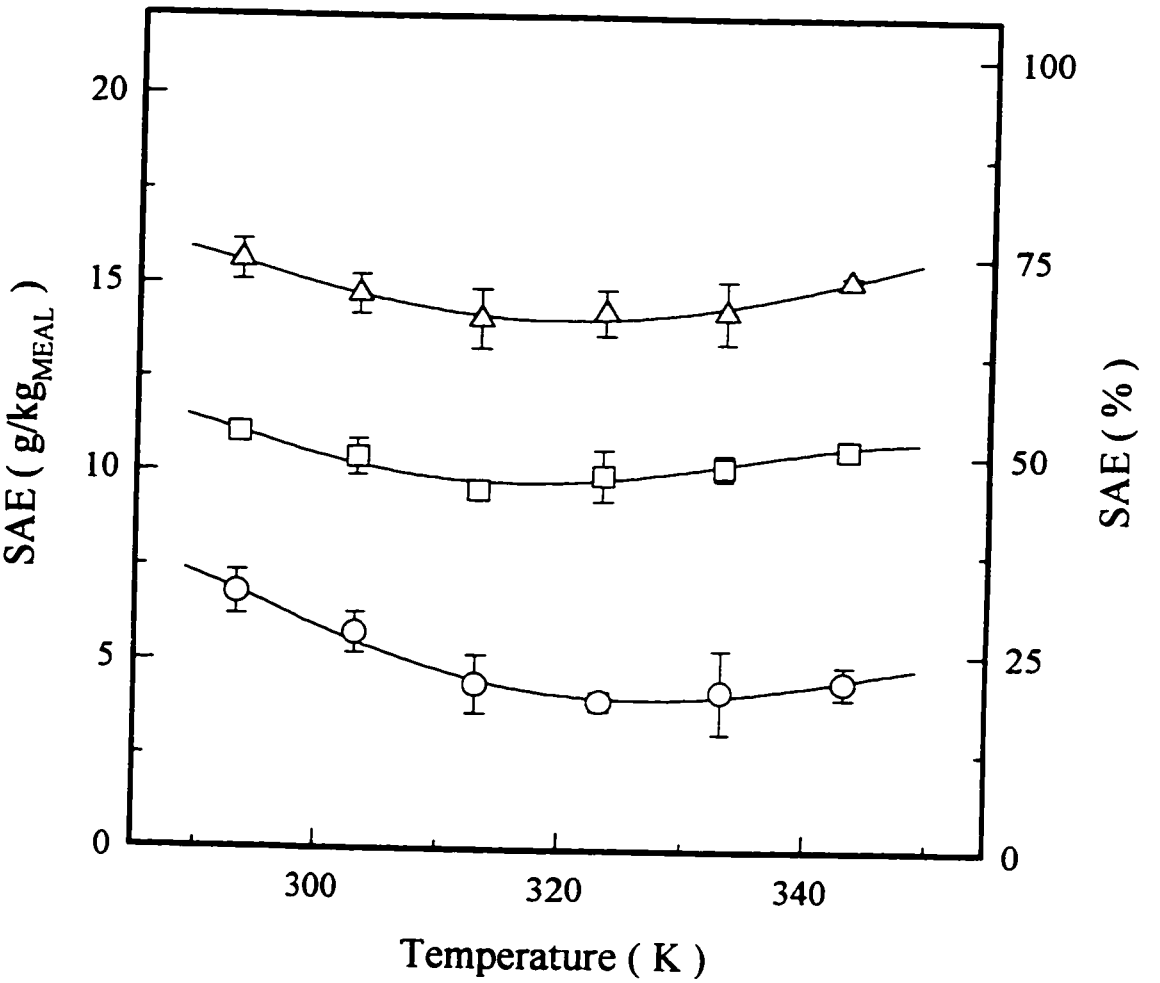


Figure 9-14. Effect of temperature on the decrease of SAE content in CM after 1 hour of treatment at pH 4.5 for a meal concentration of 20.0% (w/v): (Δ) deactivated enzyme; (□) 1.0 nkat/g; (○) 20.0 nkat/g.

This phenomenon could be explained by an increase in the enzyme stability in the presence of CM. On the other hand, the relatively weak response observed in the rate of the process when the temperature was increased up to 50°C indicated that the presence of CM could have prevented the enzyme from attaining the same favorable stereo-configuration as the one apparently present in the model enzymatic system. A similar phenomenon of a weak enzyme response to the increase in temperature is observed when enzymes are immobilized on solid supports. For instance, the inducible form of laccase from *T. versicolor* immobilized on glass beads [Rogalski *et al.*, 1990] showed such a temperature response. Expressing the enzymatic activities in terms of a percentage of the activity at the optimum temperature in the model system, and the rates of SAE decrease as a percentage of the rate at 50°C, allows a direct comparison between the effect of temperature on the enzymatic reaction rates in the CM and model enzymatic systems (Figure 9-15). For the purpose of comparison, the results obtained by Rogalski *et al.* [1990] with free and with immobilized on glass beads laccase are also presented. The results from both projects representing the free-enzyme systems are in good agreement. The results from the CM system also showed some resemblance to those obtained with the immobilized laccase, indicating that the hypothesis of the immobilization of the enzyme on CM may be correct. Furthermore, Figure 9-15 showed that in the presence of CM, the enzyme was apparently immobilized to a higher degree, comparing to the glass beads immobilization. However, since the two enzymes are different a general conclusion can not be drawn. The immobilization effect observed in the CM system could either be caused by the adsorption of the enzyme onto the canola meal particles, or by the protective action of CM proteins, or both. The stabilizing effect of proteins on the thermal stability of enzymes is well known. To determine which of these phenomena were responsible for the observed behavior of the enzyme, tests examining the effects of CM proteins and CM particles on the thermal stability of the enzyme were performed. The results obtained are discussed further in the text (section 9.5).

9.4.6 Effects of particle size-internal mass transfer

The results presented in Figure 9-7 suggested that the rate of decrease in phenolic content may be mass-transfer limited. According to these results, the latter stages of the enzymatic process were limited by the extraction of SAE from the meal matrix. From the modeling point of view, it

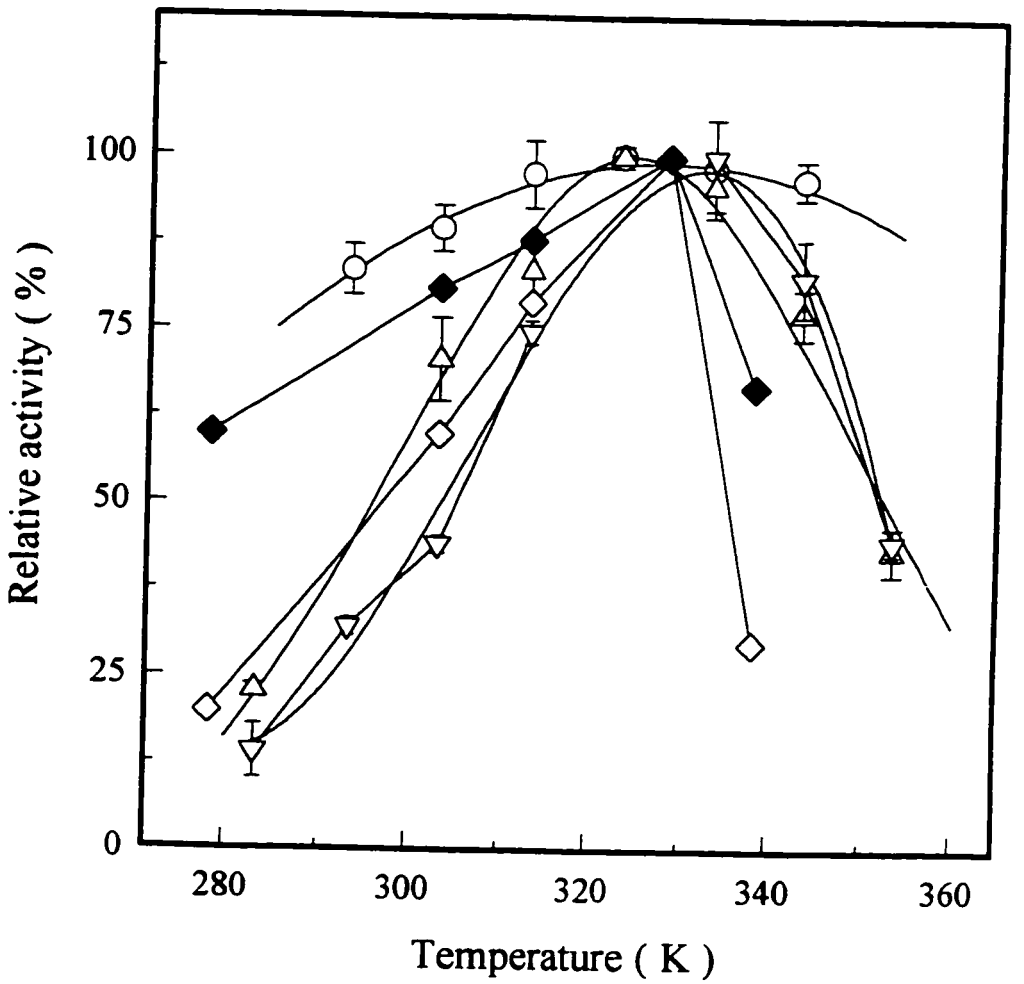


Figure 9-15. Effect of temperature on the rates of enzymatic transformation in the model and canola meal systems. This work: (O) - canola meal system; model system using: (Δ) - SIN, (∇) - SA; data from Rogalski *et al.* [1990] obtained with SA: (◊) - free enzyme; (◆) - immobilized enzyme.

was of interest to determine whether the model should account for the meal particle size distribution, or if the treated meal should be considered as a uniform material. To investigate this, the fractions of CM obtained from sieve analysis were subjected to the enzymatic treatment. Only the fractions with a diameter larger than 600μm were considered separately. The remaining fractions (Table 9-1) were combined since the hydrodynamic conditions for the enzymatic process decline significantly with a decrease in the particle diameter. A drastic increase in the buffer-enzyme uptake by CM was observed for the smaller fractions, which resulted in a stagnant meal-buffer slurry. Such a condition did not favor either the extraction of the SAE from the meal or their enzymatic transformation.

The results regarding the dynamics of SAE decrease in each of the analyzed fractions were not conclusive (Figure 9-16). For the fractions with diameters smaller than one millimeter, the results in general followed the expected trend: the smaller the particle diameter the faster the rate of SAE decrease. However, the opposite pattern was observed when the results regarding the fractions with the diameter bigger than 1.0 mm were considered. The decreases in SAE content observed for the smallest and largest particles were practically the same. It should be pointed out that the results presented in Figure 9-16 are expressed in terms of the percentage of the initial SAE content for each fraction since the initial SAE content in each fraction was different. This allowed for easier comparison of the results than if the results were expressed in terms of the mass concentration.

Although, some of the results were not in accordance with what was expected, it is logical to assume that the particle size influenced the extraction of the SAE from the meal, as shown by the data obtained using the smaller particles. Furthermore, since the meal is composed mostly of fractions smaller than one millimeter, it was decided to include the effect of particle size into the formulated model.

9.4.7 Effect of agitation - external mass transfer

To determine whether the enzymatic process depends on the external mass transfer step, the effect of intensity of agitation on the extent of the reduction in SAE content was also investigated. The agitation of CM-buffer slurry during the enzymatic treatment not only caused a

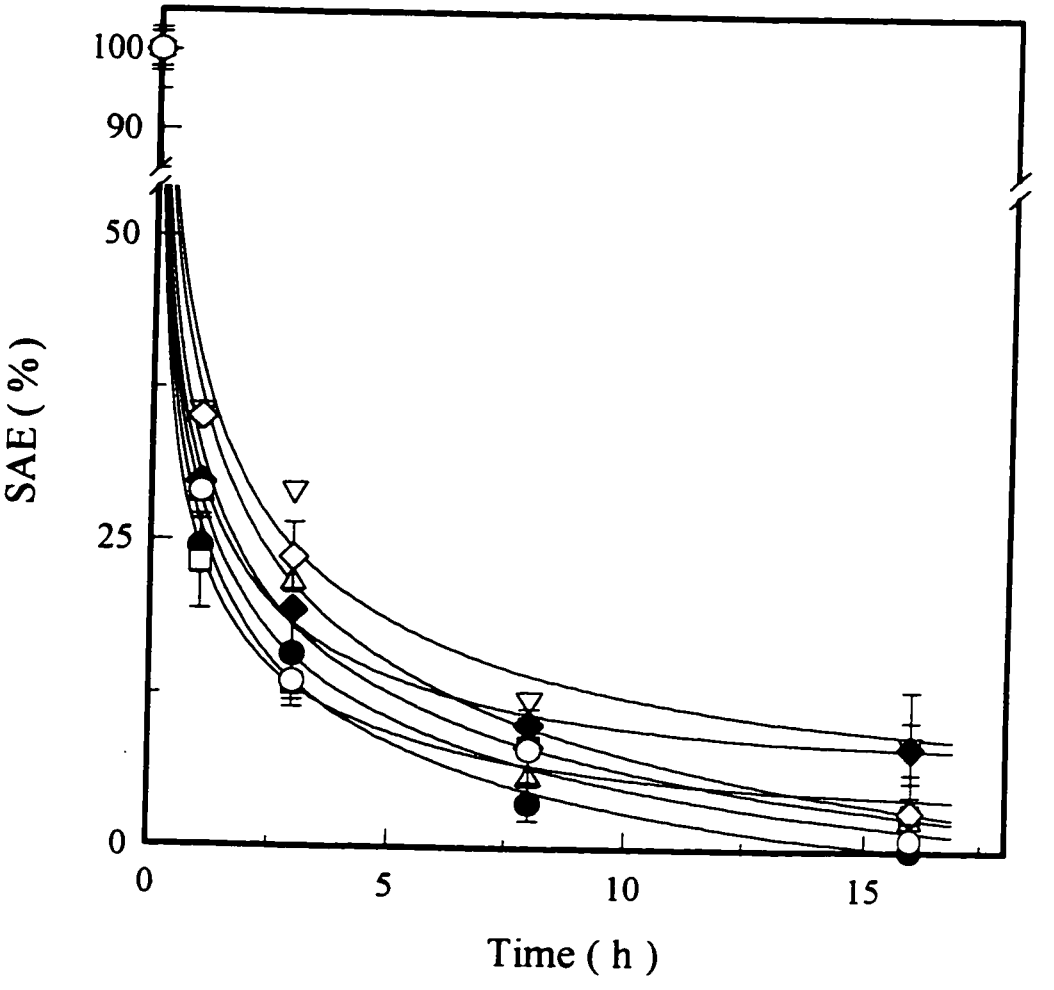


Figure 9-16. Effect of particle size on the decrease in SAE content of CM at 50°C, enzyme and meal concentration of 4.0 nkat/g and 10.0% v/w, respectively: (●) < 600μm; (□) 600÷710 μm; (Δ) 710÷841 μm; (▽) 841÷1000 μm; (◊) 1.0÷1.2 mm; (◆) 1.2÷1.4 mm; (○) >1.4 mm.

more efficient extraction of SAE from CM, by eliminating the mass transfer resistance in the boundary layer, but it also provided means for regeneration of oxygen, the concentration of which was constantly decreasing in the continuous phase due to the enzymatic reaction.

The results obtained showed (Figure 9-17) that the initial rate of the process was 2.2 times faster in the system that was agitated at the rate of 12 rpm than in the non-agitated system. However, when the intensity of the agitation was increased tenfold, no additional increase in the rate of the process was observed. In the non-agitated system, the CM particles formed a sediment where only the top layers could have been extracted to a higher degree. This extraction step was occurring because of the presence of a bigger driving force for this process, due to the decrease in SAE concentration in the bulk liquid above the sediment, occurring as a result of the enzymatic reaction. By contrast, the extraction of the SAE from the bottom layers of the sediment was an example of an extraction into a solution of limited volume. In such a case, the extraction process was governed by the local equilibrium between the concentrations of SAE in the liquid and in the CM particle. The enzymatic reaction, which under normal conditions, causes the presence of the driving force for extraction, no longer took place after the oxygen concentration in the liquid was locally depleted. Oxygen could not diffuse down through the CM sediment at a normal rate, since its concentration was decreasing due to the enzymatic reactions taking place above, and in the upper levels, of the sediment. Thus, without agitation, the overall rate of the enzymatic process was significantly suppressed due to the slow upwards diffusion of the extracted SAE and a slow reaction resulting from the low oxygen concentration at the bottom of sediment.

The results presented in Figure 9-17 showed that the external mass transfer step does not have to be included in the formulated model, providing the system is considered being perfectly mixed. In other words, the above results together with the data regarding the effect of particle size, showed that the new enzymatic process can be characterized by high Biot numbers.

9.4.8 Effect of oxygen concentration

The results obtained in the model enzymatic system showed that the enzymatic transformations of sinapic acid and sinapine are based on the conversion of 4 moles of these substrates for each mole of oxygen. To determine whether the same stoichiometry holds in the CM

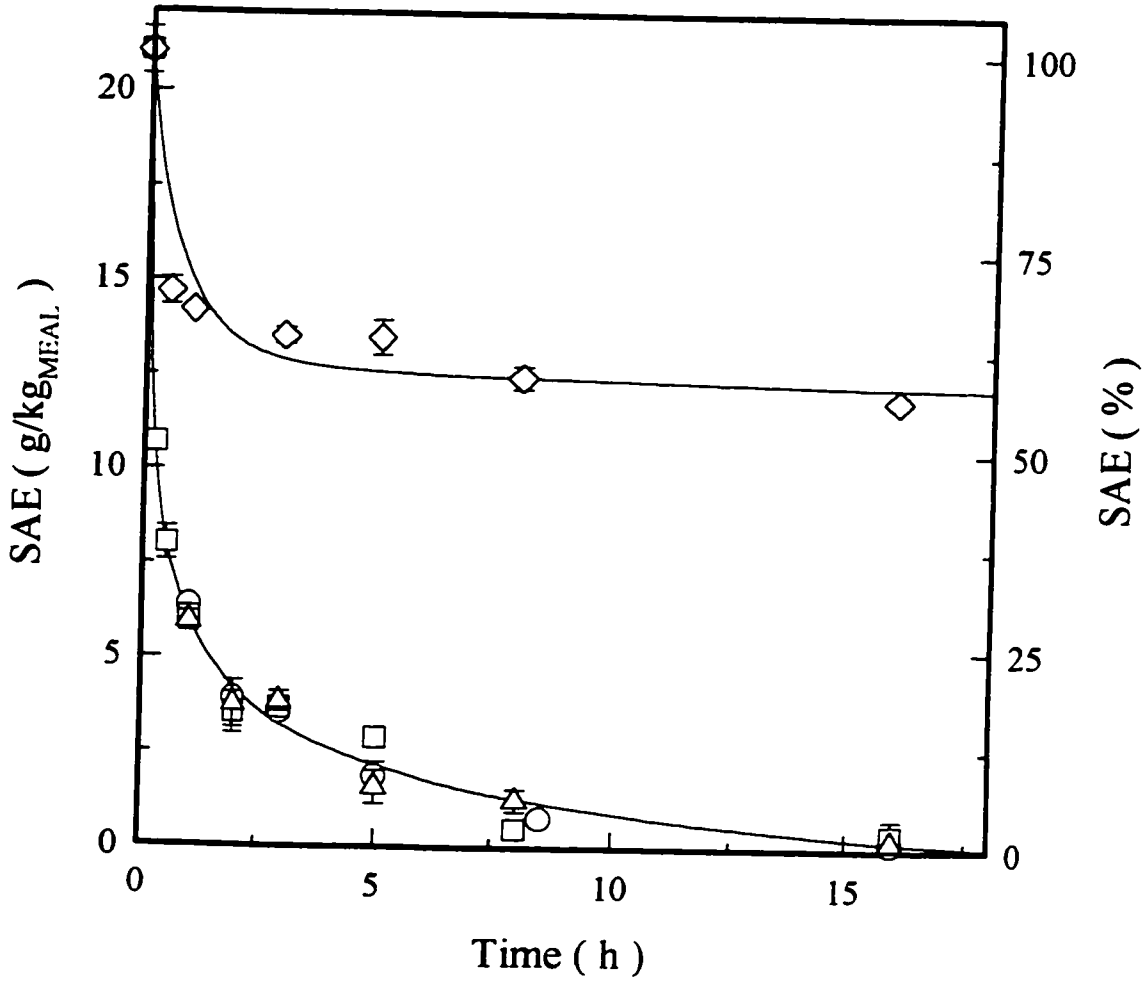


Figure 9-17. Effect of the intensity of agitation on the decrease in SAE content of CM at 50°C, enzyme and meal concentration of 4.0 nkat/g and 10.0% (w/v), respectively: (◊) no agitation; (Δ) 120 rpm; (○) 36 rpm; (□) 12 rpm.

system, experiments were performed which focused on the effect of the total amount of oxygen present in the system on the amount of SAE enzymatically decreased. Different amounts of oxygen were obtained by changing the volume of a head-gas space in the reactor, in which the enzymatic treatment was being carried out. This was done by varying the volume of the continuous phase and keeping the meal concentration constant. The results obtained (Table 9-6) showed that all of the SAE present in the system were converted when the ratio of the initial amounts of SAE and oxygen was less than 4. When this ratio was equal to and/or bigger than 4, the transformation of SAE was not complete. These results were expected, at least in the case of ratio equal to 4, because of the anticipated presence of additional enzymatic reactions involving oxygen and the products of the enzymatic conversion of SAE and/or some other compounds of phenolic origin, *i.e.*, tannins. On the other hand, the larger than expected amounts of SAE converted in the system where the initial oxygen concentration was 14 times lower than the concentration of SAE suggested that oxygen was reinstated during the enzymatic process.

Table 9-6. Effect of available oxygen on the amount[a] of SAE transformed after enzymatic treatment of 10.0% meal suspension at 25°C

Initial	Volume [mL]		oxygen [μmol]			Process	SAE [μmol]		Ratio of SAE/O_2	
SAE [μmol]	head space	liquid	head[b]	liquid[c]	total	time [h]	liquid	transformed	initial	final
29.19	7.2	6.0	11.98	1.57	13.55	1	1.05 (1.01)	28.83 (0.03)	2.15	2.13
						4	0.67 (0.15)	29.00 (0.02)		2.14
						8	0.0	29.00 (0.01)		2.14
38.92	5.0	8.0	8.32	2.10	10.42	1	6.73 (0.68)	28.45 (0.72)	3.73	2.73
						4	4.22 (0.03)	30.92 (0.03)		2.97
						8	5.30 (0.30)	31.54 (0.54)		3.02
48.65	2.8	10.0	4.66	2.62	7.28	1	14.56 (1.41)	27.33 (0.28)	6.68	3.75
						4	14.48 (3.51)	27.50 (2.57)		3.78
						8	14.36 (3.56)	30.19 (2.06)		4.14
58.38	0.65	12.0	1.08	3.15	4.23	1	26.26 (0.13)	23.07 (2.67)	13.80	5.45
						4	22.57 (1.51)	23.88 (2.69)		5.64
						8	20.72 (0.58)	27.58 (0.87)		6.52

[a] - calculated using molecular weight of sinapine bisulfate
[b] - assuming ideal gas behaviour at atmospheric pressure
[c] - calculated using the solubility of oxygen at 25°C
values in brackets represent standard deviations

9.5 Enzyme stability in the presence of canola meal

As discussed in the preceding sections, there was some evidence that the canola meal had a significant effect on the thermal stability of the enzyme. To examine the hypothesis that the thermostability of the enzyme increases in the presence of canola meal, the enzyme preparation was incubated in 10.0% (w/v) suspension of CM under different conditions including: various temperatures, pH, protein concentrations, and meal and protein type.

9.5.1 Effect of temperature

At first the effect of temperature on the stability of the enzyme in the presence of CM was considered. Changes in the activities of the incubated enzyme obtained at various temperatures of incubation are presented in Figure 9-18. The results showed that the stability of enzyme increased substantially in the presence of CM. The half-life times for the enzyme incubated with CM at 50, 60, 70, and 75°C were 45, 10.5, 3.5, and 1.5 hours, respectively. At 50°C the decay in the activity was 4 times slower than the one observed when the enzyme was incubated in the citrate-phosphate buffer alone, and even after a 150 hour-long incubation, the enzyme retained 10.0% of its initial activity. Considering the stability of the enzyme at 60°C observed in the presence of CM and that at 50°C in the buffer, it is noticed that the meal presence compensates for the 10°C increase in the incubation temperature.

9.5.2 Effect of pH

Bearing in mind that pH affected stability of the enzyme in the model system [Łącki and Duvnjak, 1996a], it was decided to study the effect of this variable on the enzyme stability in the presence of CM.

Three pH levels of the system were tested: 3.2, 4.0, 5.0. The results obtained (Figure 9-19) showed that in the presence of CM the enzyme was the most stable at pH 5.0. Its stability decreased with a decrease in the system pH. The change in pH from 5.0 to 3.2 produced a similar deactivation effect as an increase in the incubation temperature from 50°C to 75°C at pH 5.0.

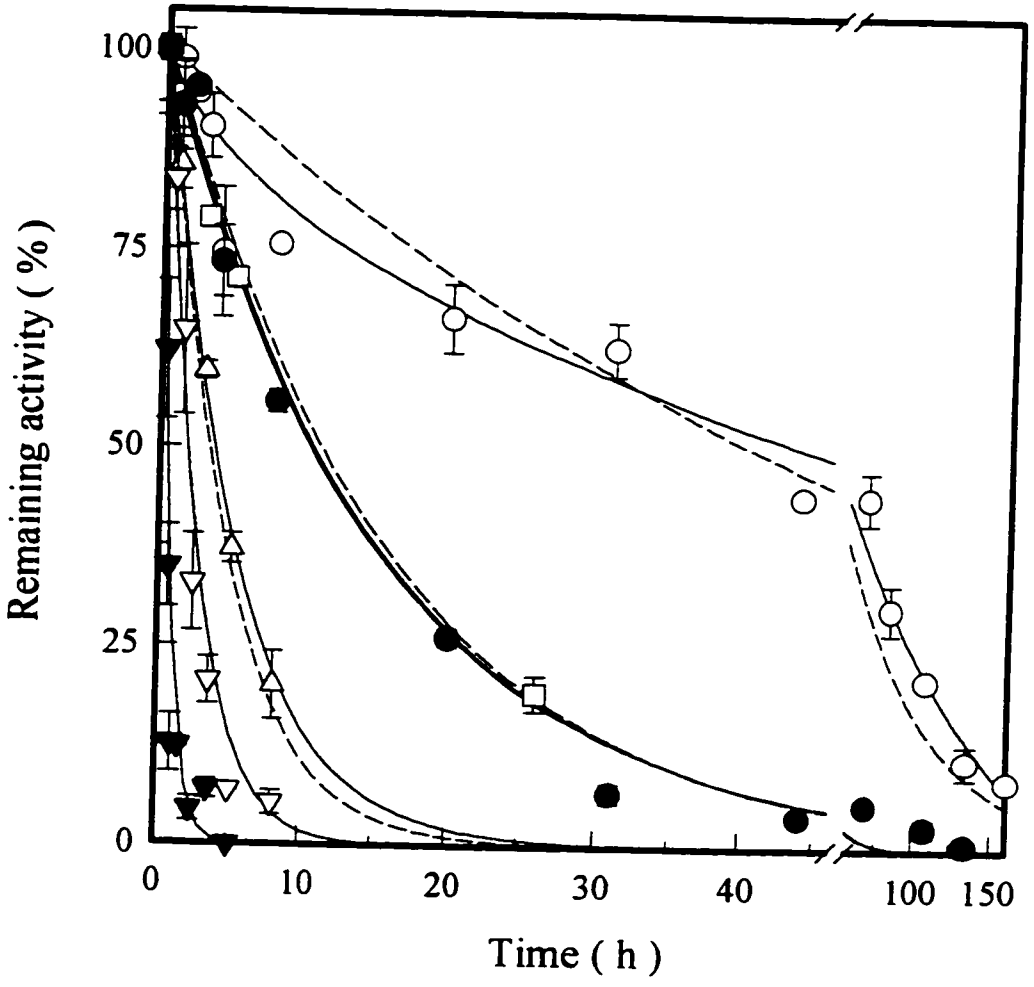


Figure 9-18. Effect of temperature on the stability of the enzyme in the presence and absence of CM at pH 5.0: 10.0 % (w/v) meal suspension: (O) 50°C; (□) 60°C; (Δ) 70°C; (▽) 75°C; 0.0 % (w/v) meal suspension: (▼) 75°C; (●) 50°C.

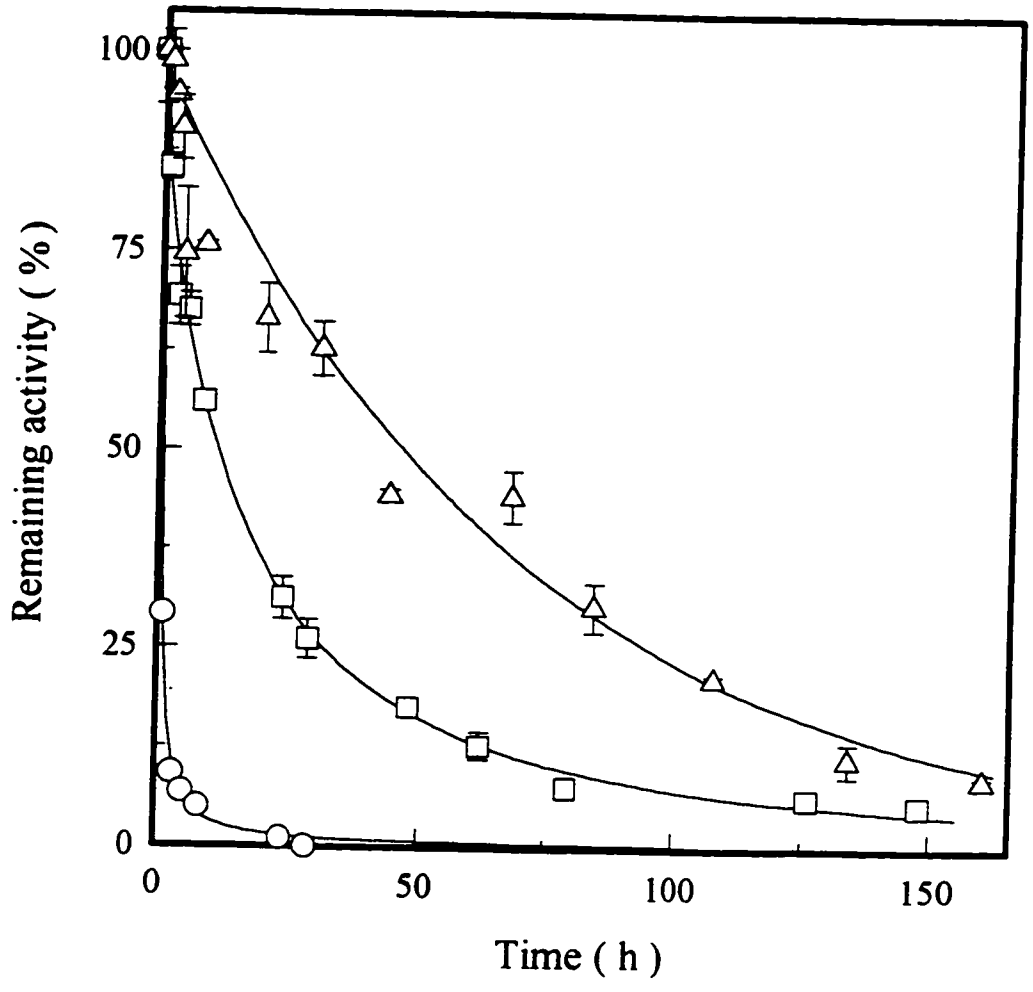


Figure 9-19. Effect of pH of the system on the stability of the enzyme in the presence of CM at 50°C: (O) pH 3.2; (□) pH 4.0; (Δ) pH 5.0.

Since the decrease in pH of the system to 3.2 resulted in a lower concentration of proteins in the continuous phase (Figure 9-13), the effect of the concentration of canola meal proteins on the stability of the enzyme was also considered.

9.5.3 Effect of protein concentration

To investigate the effect of canola meal proteins on the enzyme stability, the enzyme was incubated in protein solutions at two concentrations: 12.2 mg/mL and 9.5 mg/mL. These concentrations represented the concentration of CM proteins measured in the 10.0% (w/v) meal-buffer suspension at pH 5.0 and 3.2, respectively. To determine whether the type of proteins had any effect on the enzyme stability, the enzyme was also incubated in bovine-albumin solutions of the identical concentrations as these used in tests with CM proteins. The obtained results showed (Figure 9-20), that the enzyme was the most stable when incubated at a higher concentration of CM proteins. A 20.0% decrease in the concentration of proteins resulted in a 5.0% decrease in the enzyme stability. This effect was more obvious in the later stages of the incubation process. The presence of bovine-albumin, regardless of its concentration, did not have any effect on the stability of the enzyme compared to its stability in the citrate-phosphate buffer alone. These results showed that both the protein concentration and their type affect the stability of the enzyme.

9.5.4 Effect of canola meal particles

The results regarding incubation of the enzyme at 50°C with CM (Figure 9-18) and with CM proteins (Figure 9-20) showed, that the stability was higher in the presence of CM particles. To determine whether this result was caused either by the CM proteins found on the surface of particles or by some other CM components, the enzyme was incubated with a protein-free CM. Such a meal was obtained by extracting all the meal proteins at pH 12. The results from this experiment are presented in Figure 9-21 where they are directly compared with the previously shown results regarding the incubation of the enzyme at 50°C. The results showed that the enzyme incubated with the protein-free CM was more stable than the enzyme incubated in the solution of CM proteins, but less stable than the one incubated in the presence of untreated CM (Figure 9-21).

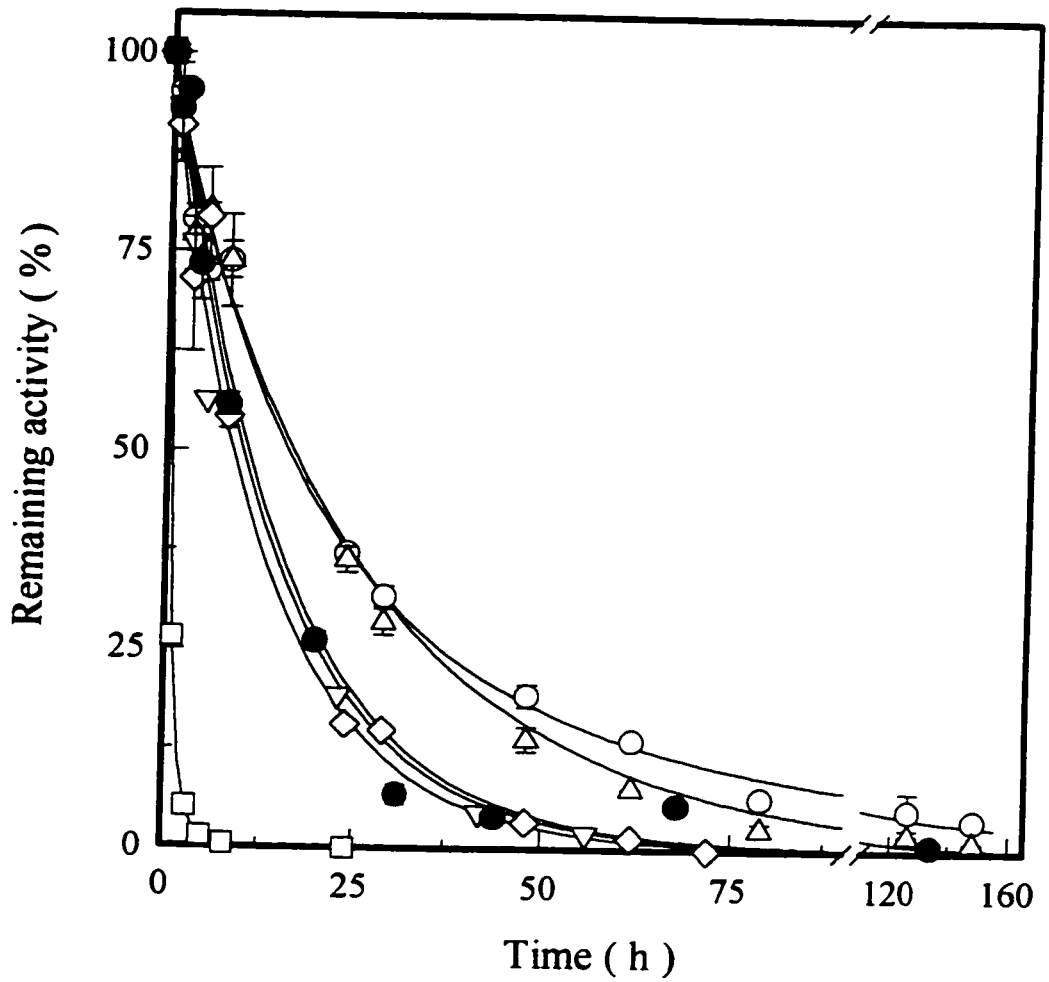


Figure 9-20. Effect of the type of proteins, their concentrations and pH of the system on the stability of the enzyme at 50°C. System pH 5.0: (○) 12.2 mg/mL of CM proteins; (△) 9.5 mg/mL of CM proteins; (▽) 12.2 mg/mL of bovine-albumin, (◇) 9.5 mg/mL of bovine-albumin; (●) no proteins. System pH 3.2: (□) 9.5 mg/mL of CM proteins.

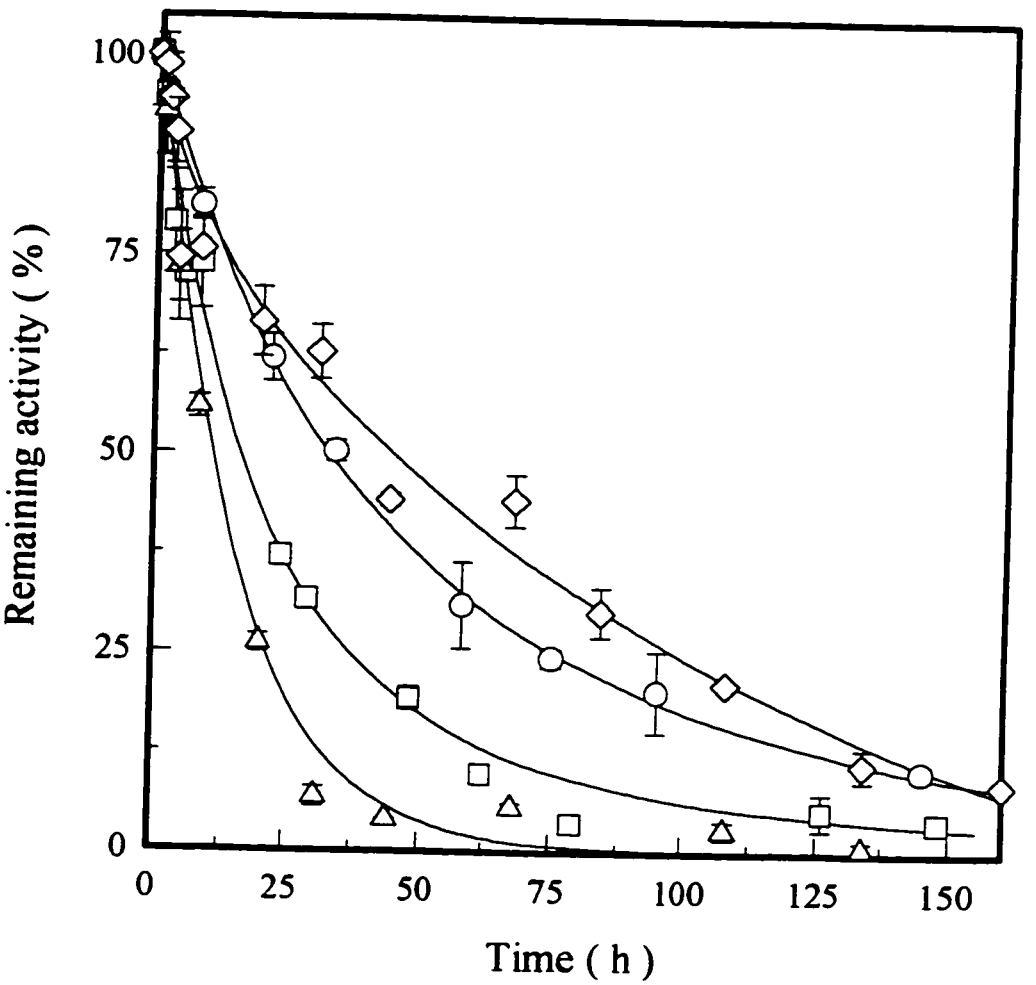


Figure 9-21. Stability of the enzyme at 50°C and pH 5.0 in the presence of: 10% (w/v) CM (◊); 10% (w/v) protein-free CM (O); 12.2 mg/mL CM proteins (□); citrate phosphate buffer 0.0 mg/mL CM proteins (Δ).

Therefore, it can be concluded that the protective action of CM particles was not related to the CM proteins found in the meal after the buffer extraction at pH 5.0.

The protective action of CM particles, regardless of the pH (Figure 9-19 and Figure 9-20) was also observed when a long term incubation of the enzyme at room temperature in the presence of CM was investigated (Figure 9-22). The enzyme showed a higher stability when incubated with normal CM than when incubated in the buffer alone or in the solution of CM proteins.

The results obtained from the enzyme stability studies showed that the increase in the thermal stability of the enzyme observed in the presence of CM was caused by the protective actions of CM particles and proteins. Accordingly, the time changes in the concentration of the active enzyme during the enzymatic treatment of CM should be described by models that take both of these phenomena into account. In this work, only a semi-empirical formula describing the changes in the concentration of active enzyme incubated with CM was proposed. Formulation of a full mechanistic model would require a larger amount of specific experimental data.

9.5.5 Modeling of enzyme deactivation

Modeling of the enzyme deactivation was based only on the data regarding the effect of temperature on the enzyme stability (Figure 9-18). This effect was of the greatest interest in terms of an eventual implementation of the deactivation model into the mathematical description of the enzymatic process to decrease the phenolic content in CM. The effect of pH was not considered since the enzymatic treatment of CM was carried out at constant pH. The effect of different protein concentrations in the continuous phase was also not included in the deactivation model, since the enzymatic process was always carried out in the presence of CM particles, which showed a stronger effect on the stability of the enzyme than the proteins themselves. Consequently, only the data presented in Figure 9-18 were used to estimate the parameters in the proposed models. Several models were tested, including mono- and multi- exponential functions combined with logistic expressions. Among them the model given by Eq.(9.4) was chosen, since it represented a reasonable deactivation mechanism.

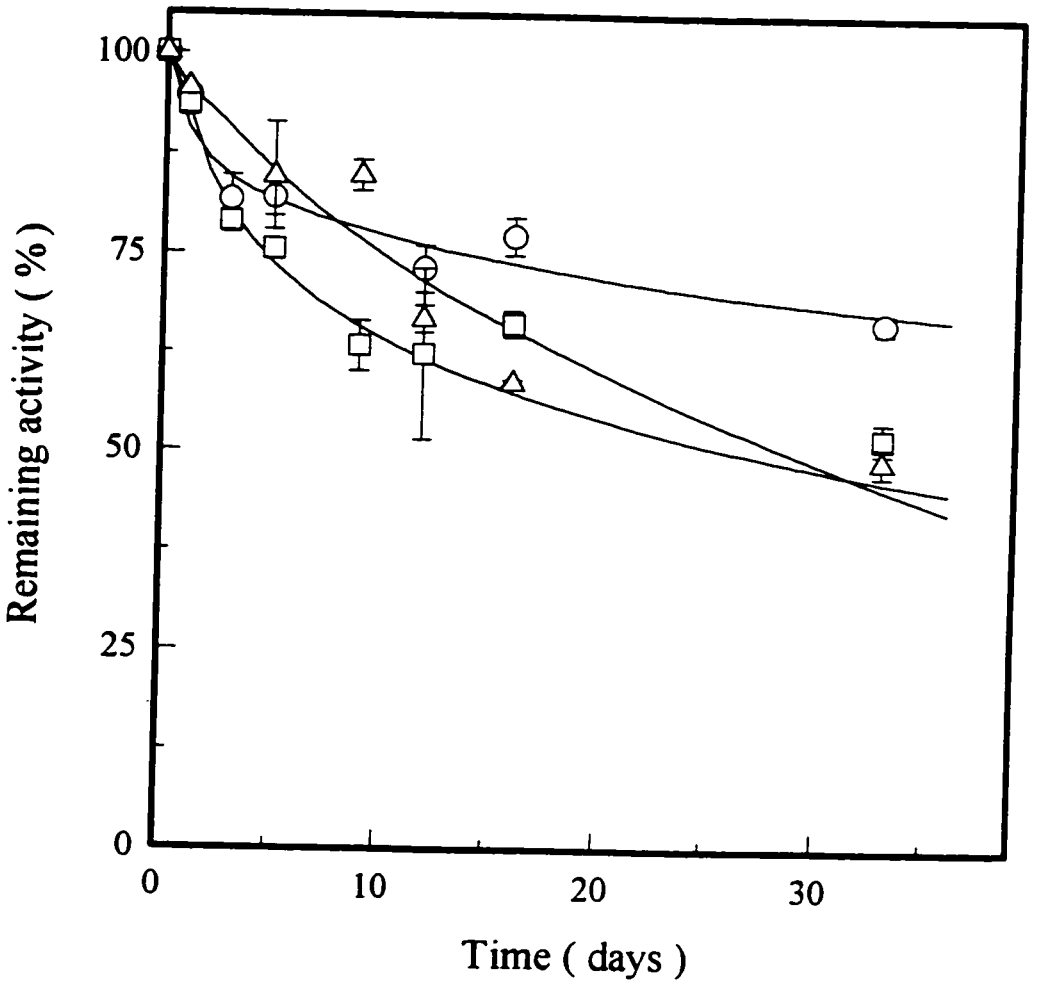


Figure 9-22. Stability of the enzyme at room temperature in the presence and absence of CM at pH 5.0: (O) 10 % (w/v) CM suspension; (□) 12.2 mg/mL of CM proteins; (Δ) citrate-phosphate buffer.

$$E_{CM} = E_0\left[e^{-r_2 t} + \frac{r_1}{r_2 - r_3}(e^{-r_3 t} - e^{-r_2 t})\right] \tag{9.4}$$

where: E_0 and E_{CM} represent the initial concentration of the active enzyme and the concentration of the active enzyme after time t of incubation in the presence of CM, respectively; r_1, r_2 and r_3 represent model parameters.

It can be shown (Appendix B) that Eq.(9.4) gives the concentration of active enzyme if the mechanism of deactivation is as follows: upon exposure to higher temperatures, the enzyme irreversibly transforms to an inactive form *via* two pathways: one direct and the other involving the formation of an intermediate active form that will eventually also irreversibly deactivate but at different rate. All the reaction steps in the proposed mechanism are first order, with the rate constants k_1, k_2, k_3, respectively, for the direct deactivation (step 1), the formation of the intermediate form (step 2), and the deactivation of the intermediate form (step 3).

The parameters in Eq.(9.4) were estimated using a non-linear least-squares method. The rate constants can be calculated from the model parameters, r_i, according to the relationships given in the footnote of Table 9-7. A dependence of the rate constants on temperature, after exclusion of the data at 308 K, showed a desirable pattern represented by a linear relationship between the natural logarithm of the rate constant and the inverse of temperature. Respective frequency factors, k_{0i}, and energies of activation, E_i, calculated using the transition state theory are given in Table 9-7. The results predicted by the model, using values of the parameters calculated from the frequency factors and energies of activation, were shown as dashed lines in Figure 9-18.

Table 9-7. Values of the respective rate constants obtained from the estimates of the parameters in Eq.(9.4)[a]

Parameter	Temperature [K]					$k_{0,i}$	E_i
	308	323	333	343	348	[h^{-1}]	[kJ mol^{-1}]
k_1	0.0144	0.0026	0.0138	0.0316	0.0789	1.20×10^{14}	118.392
k_2	0.0038	0.0158	0.0632	0.1693	0.3578	3.86×10^{13}	110.652
k_3	0.0002	0.0354	0.1091	0.6897	1.3218	1.31×10^{18}	137.013

[a] - relationship between rate constants and parameters in Eq.(9.4): $k_1 = r_1$; $k_2 = r_2 - r_1$; $k_3 = r_3$; $k_{0,i}$ frequency factor; E_i, energy of activation.

9.6 Mathematical modeling of the enzymatic process to decrease the phenolic content in canola meal

In this section the mathematical model describing the investigated enzymatic process to decrease the phenolic content in commercial canola meal will be presented and discussed. The results of the estimation of the model parameters will be given, and some simulation results focusing on a few aspects of the process will be presented.

9.6.1 Derivation of the model

Considering the experimental results presented in the previous sections, the following process variables were taken into account in the mathematical model: the initial meal, enzyme and oxygen concentrations, the size distribution of the meal particles, and the temperature of the process.

In general, the mass transfer phenomena that needed to be considered in modeling of the enzymatic process to decrease the phenolic content in canola meal are: diffusion of the SAE within the canola meal particle (intra-particle diffusion); diffusion of SAE in the pores of CM (intra-pore diffusion); desorption of SAE from the surface of CM according to linear or non-linear isotherm; diffusion of the SAE through the stagnant film (boundary layer) surrounding the particle (external mass transfer); and accumulation of the SAE in the bulk liquid. Each of these steps, with the exception of intraparticle diffusion, can be accompanied by the enzymatic reaction.

For the development of the model it was assumed that the enzymatic reaction takes place only in the liquid, and that the intrapore diffusion is negligible. In addition, the following assumptions were made:

1. The system is isothermal and perfectly mixed;
2. CM particles are spheres, and their size and geometry do not change during the process;
3. Effective diffusivity in the CM, D_{eff}, is constant;
4. Negligible diffusional resistance in the external film, *i.e.*, high Biot number;
5. Density of the continuous phase is constant;
6. The initial distribution of SAE in the meal is uniform;

7. At equilibrium, the apparent concentration of SAE in the meal and their concentration in the bulk liquid are related by the partition coefficient;
8. The enzymatic conversion of the SAE extracted from CM is described by a single reaction proceeding according to Theorell-Chance Bi-Bi mechanism;
9. Additional enzymatic reactions involving the products of SAE transformations, P_{CM}, are described by a single reaction step that proceeds according to the Theorell-Chance Bi-Bi mechanism;
10. The gas phase obeys the ideal gas law and the concentration of oxygen in the continuous phase can be calculated from Henry's law. The changes in the pressure of the gas phase are small and are not considered. The effect of salts on the oxygen concentration in the bulk liquid is also not considered.

9.6.1.1 Model equations

The enzymatic decrease of the phenolic content in the spherical particles of CM in the system composed of three phases: gas, liquid and solid, is described by the following system of equations representing differential mass balances of oxygen, SAE and P_{CM} in respective phases:

Gas phase:

$$V_g \frac{dC_{O_2}^g}{dt} = -V_l k_L^{CM} a\left(C_{O_2}^* - C_{O_2}^l\right) \tag{9.5}$$

The initial conditions for Eq.(9.5) is:

$$C_{O_2}^g = C_{0,O_2}^g \;\; ; \;\; t = 0 \tag{9.6}$$

Solid phase:

$$\frac{D_{eff,i}}{r_i^2}\frac{\partial}{\partial r}\left(r_i^2 \frac{\partial w_i}{\partial r}\right) = \frac{\partial w_i}{\partial t} \;\; ; \; 0 \le r_i \le R_i \; ; \; t \ge 0 \tag{9.7}$$

$$3M_{S,i}\frac{D_{eff,i}}{R_i}\frac{\partial w_i}{\partial r} = N_{0,i} \;\; ; \;\; r_i = R_i \; ; \; t > 0 \tag{9.8}$$

The initial conditions for Eq.(9.7) and Eq.(9.8) are:

$$w_i = f_i(r) = w0_i \;\; ; \; 0 \le r_i \le R_i \;\; ; \;\; t = 0 \tag{9.9}$$

$$w_i = f\left(C_{SAE}\right) = K_{iso} C_{SAE} \;\; ; \; r_i = R_i \;\; ; \;\; t > 0 \tag{9.10}$$

$$\frac{\partial w_i}{\partial r} = 0 \;\; ; \;\; r_i = 0 \;\; ; \;\; t > 0 \tag{9.11}$$

Liquid phase:

$$\frac{dC^l_{O_2}}{dt} = k_L^{CM} a\left(C^*_{O_2} - C^l_{O_2}\right) - r_{O_2} \tag{9.12}$$

$$\frac{dC_{SAE}}{dt} = -\frac{1}{V_l}\sum_{i=1}^{J} \frac{N_{0,i}}{Mw_{SAE}} - r_{SAE} \;\; ; \;\; t > 0 \tag{9.13}$$

$$\frac{dC_P}{dt} = r_{SAE} - r_P \tag{9.14}$$

The initial conditions for Eqs.(9.12), (9.13), and (9.14) are:

$$C_{SAE} = C_{SAE,0} \;\; ; \;\; C_{P_{CM}} = 0 \;\; ; \;\; C^l_{O_2} = C^l_{O_2,0} \;\; ; \;\; t = 0 \tag{9.15}$$

Eq.(9.10) shows that the boundary condition for Eq.(9.7) is time dependent and, therefore, in addition to the numerical solution of a second order partial differential equation, PDE, the solution to the above system of equations requires a knowledge of the time concentration profile of the SAE in the bulk liquid. Considering that the latter was not known, another approach was considered in this work. Following the work of Papathanasiou *et al.* [1987] concerning the dynamic behaviour of immobilized cell/enzyme bioreactors, the second order PDE representing the intraparticle mass balance, Eq.(9.7), was decomposed into a set of first order ODEs, that together with ODEs representing the mass balances in the bulk liquid, Eq.(9.13), and the gas-phase, Eq.(9.5), could be solved using one of the standard numerical methods. The surface concentration time profile obtained in this way is used to obtain the intraparticle concentration distribution.

The decomposition of the PDE involves the application of *Duhamel's theorem* to obtain the solution of Eq.(9.7) for the time dependent boundary condition (Eq.(9.10)), using the solution of the same equation for the constant boundary condition. Details of the applied procedure are presented in Appendix B, section B-4. The final set of ODEs describing the enzymatic process for decreasing the phenolic content in CM is as follows:

$$\frac{dX_{O_2}^{gas}}{dt} = -\frac{RT}{P}\frac{V_l}{V_g}k_L^{CM}a\left(C_{O_2}^{*} - C_{O_2}^{l}\right) \tag{9.16}$$

$$\frac{dC_{O_2}^{l}}{dt} = -\frac{\beta k_{s,CM}E_{CM}C_{O_2}^{l}\left(K_{m,P_{CM}}C_{SAE} + K_{m,SAE}C_{P_{CM}}\right)}{(C_{O_2}^{l} + K_{m,O_2,CM})(K_{m,P_{CM}}C_{SAE} + K_{m,SAE}C_{P_{CM}}) + K_{m,SAE}K_{m,P_{CM}}(C_{O_2}^{l} + K_{i,O_2,CM})} + \\ + k_L^{CM}a\left(C_{O_2}^{*} - C_{O_2}^{l}\right) \tag{9.17}$$

$$\frac{dC_{SAE}}{dt} = \frac{-\frac{6}{Mw_{SAE}}\frac{M_S}{V_l}\sum_{i=1}^{J}\frac{x_i D_{eff,J}}{R_i^2}\left[\sum_{n=1}^{\infty}\left(w0_i - K_{uso,J}C_{SAE,0}\right)e^{-k_{n,J}t} - \sum_{n=1}^{n_0}\Psi_{n,J}\right]}{1+\frac{M_S}{V_l}B_{uso,J}\left(1-\frac{Sl_{n0}}{Sl_{\infty}}\right)} - \\ - \frac{\frac{m\beta k_{s,CM}K_{m,P_{CM}}E_{CM}C_{O_2}^{l}C_{SAE}}{(C_{O_2}^{l} + K_{m,O_2,CM})(K_{m,P_{CM}}C_{SAE} + K_{m,SAE}C_{P_{CM}}) + K_{m,SAE}K_{m,P_{CM}}(C_{O_2}^{l} + K_{i,O_2,CM})}}{1+\frac{M_S}{V_l}B_{uso,J}\left(1-\frac{Sl_{n0}}{Sl_{\infty}}\right)} \tag{9.18}$$

$$\frac{d\Psi_{n,J}}{dt} = -D_{eff,J}\frac{n^2\pi^2}{R_i^2}\Psi_{n,J} + B_{uso,J}\frac{dC_{SAE}}{dt}$$

$$n = 1,2,3,.....,n_0 \tag{9.19}$$

$$\frac{dP_{CM}}{dt} = \frac{m\beta k_{s,CM}E_{CM}C_{O_2}^{l}\left(K_{m,SAE}C_{P_{CM}} - K_{m,P_{CM}}C_{SAE}\right)}{(C_{O_2}^{l} + K_{m,O_2,CM})(K_{m,P_{CM}}C_{SAE} + K_{m,SAE}C_{P_{CM}}) + K_{m,SAE}K_{m,P_{CM}}(C_{O_2}^{l} + K_{i,O_2,CM})} \tag{9.20}$$

$$E_{CM} = E + E_p = E_0\left[e^{-r_3 t} + \frac{r_1}{r_3 - r_2}\left(e^{r_2 t} - e^{r_3 t}\right)\right] \tag{9.21}$$

$$\overline{w}_{tot}(t) = \sum_{i=1}^{J} x_i K_{iso,i} Mw_{SAE} C_{SAE} - \frac{Mw_{SAE}}{15} \sum_{i=1}^{J} x_i \frac{B_{iso} R_i^2}{D_{eff,i}} \left(1 - \frac{S2_{n0}}{S2_{\infty}}\right) \frac{dC_{SAE}}{dt} + \\ + \sum_{i=1}^{J} x_i w0_i \left(1 - \frac{S1_{nf}}{S1_{\infty}}\right) + \frac{6}{\pi^2} \sum_{i=1}^{J} x_i \left[\sum_{n=1}^{\infty} \frac{1}{n^2} \left(w0_i - K_{iso,i} C_{SAE,0}\right) e^{-k_{n,i} t} - \sum_{n=1}^{n_0} \frac{1}{n^2} \Psi_{n,i} \right] \tag{9.22}$$

with the following initial conditions:

$$t = 0;\ \Psi_{n,i} = 0;\ X_{O_2}^{gas} = X_{O_2,ini}^{gas};\ C_{O_2}^{l} = C_{O_2,ini};\ C_{P_{CM}} = 0;\ C_{SAE} = C_{SAE,0} \tag{9.23}$$

The equilibrium concentration of oxygen at the gas-liquid interface can be calculated from the oxygen solubility data using Henry's coefficient (Appendix B). The parameter *m* represents the stoichiometry of the enzymatic transformation of SAE and P_{CM}. The parameter $B_{iso,i}$ depends on the sorption isotherm used to describe the surface equilibrium condition and, therefore, can be either dependent or independent of the concentration of the SAE in the bulk liquid.

Bearing in mind that the total concentration of the SAE in the enzymatically treated meal was of the greatest interest, the concentration of the SAE in the solid phase was expressed as the overall-average concentration in the whole population of the meal particles (Eq.(9.22)). This approach also allowed the estimation of the model parameters to be performed using the experimental data describing the changes in the overall-average concentration of the SAE in the CM during the process.

The numerical solution of Eqs.(9.16)-(9.22) yields the dynamics of the enzymatic process for decreasing the phenolic content in CM. The accuracy of this solution depends on the accurate approximation of the infinite series solution of Eq.(9.7). As shown in Appendix B, Eqs.(9.18), (9.19), and (9.22) were derived taking into account the behaviour of the functions $\Psi_{n,i}(t)$ [Appendix B] which leads to the introduction of the approximation of the infinite series [Papathanasiou *et al.*, 1987].

9.6.1.2 Numerical solution

The numerical solution of Eqs.(9.16)-(9.22) was obtained using the Episode algorithm for solving systems of stiff ODEs. The accuracy of the numerical solution was tested against the analytical

solution obtained for two limiting cases: (1) no enzymatic reaction; and (2) the infinitely fast enzymatic reaction. For these two cases, the problem of extraction with a chemical reaction reduces to the problem of extraction of the solute into a solution of finite volume (case 1) and, to the problem of extraction with a constant, zero surface concentration (case 2).

Case 1:

The analytical solution for the problem of extraction from a sphere into a solution of the finite volume is given by [Crank, 1983]:

$$\frac{c(t)-c_\infty}{c_0-c_\infty} = -\sum_{n=1}^{\infty} \frac{6(1+\alpha)e^{-\frac{D_{eff}q_n^2}{R^2}t}}{9+9\alpha+q_n^2\alpha^2} \frac{R}{r} \frac{\sin\frac{q_n r}{R}}{\sin q_n} \tag{9.24}$$

In Eq.(9.24), parameters q_n are the non-zero positive roots of the equation:

$$\tan q_n = \frac{3q_n}{3+\alpha q_n^2} \tag{9.25}$$

where: the parameter α is defined as the ratio of the solid volume to the bulk liquid volume:

$$\alpha = \frac{V_S}{V_l} K_{LSO} \tag{9.26}$$

Eq.(9.24) expressed in terms of the average concentration is given by

$$\frac{\bar{c}(t)-\bar{c}_\infty}{c_0-\bar{c}_\infty} = \sum_{n=1}^{\infty} \frac{18\alpha(1+\alpha)}{9+9\alpha+q_n^2\alpha^2} e^{-\frac{D_{eff}q_n^2}{R^2}t} \tag{9-27}$$

Eq.(9-27) was solved with the value of α equal 1, which represents the situation when half of the solute initially present in the solid is extracted after an infinite time. In the calculations, only the first one hundred terms of the infinite series were considered. The first 15 positive nonzero roots of Eq.(9.25) were obtained using Maple v.3.0, whereas the remaining roots were predicted using a linear relationship between q_n and n, obtained by a linear regression on the actual first 15 nonzero roots.

$$q_n = 3.1139n + 0.3991 \quad R^2 = 1.0 \tag{9.28}$$

The error introduced by this procedure to the solution of Eq.(9-27) was smaller than the one when the infinite series was evaluated using the first 15 non zero roots of Eq.((9.25).

Case 2:

The analytical solution to the problem of diffusion from a sphere that is initially at a uniform concentration, c_0 , and its surface being maintained constant at zero concentration is given by [Crank, 1983]:

$$c = c_0 \frac{2R}{\pi r} \sum_{n=1}^{\infty} \frac{(-1)^{n+1}}{n} e^{-D_{eff}(n\pi/R)^2 t} \sin\left(\frac{n\pi r}{R}\right) \tag{9.29}$$

or in terms of the average concentration:

$$\bar{c} = \frac{6c_0}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} e^{-D_{eff}\left(\frac{n\pi}{R}\right)^2 t} = c_0 \left(\frac{6}{\pi^2} \sum_{n=1}^{n_f} \frac{1}{n^2} e^{-D_{eff}\left(\frac{n\pi}{R}\right)^2 t} + 1 - \frac{Sl_{n_f}}{Sl_{\infty}} \right) \tag{9.30}$$

The results obtained for both limiting cases using the analytical solutions were compared with those that were obtained by the numerical solution of Eqs.(9.16)-(9.23) for the following process conditions; *case 1*: zero enzyme concentration, M_s=0.5, V_f=5, K_{iso}=10, *i.e.*, α=1; *case 2*: the enzyme concentration was set to E_{CM} = 100000 nkat/mL, k_La = 100000 h^{-1}, X_{im} = 100, *i.e.*, infinitely fast reaction no oxygen limitation.

Figure 9-23 shows that the analytical and the numerical solutions were virtually the same for both limiting cases, with the maximum errors less than 1.2% for *case 1* and no error for *case 2.*

9.6.2 Additional aspects of the modeling of the enzymatic process

In general, the number of differential equations that need to be solved simultaneously to obtain the numerical solution of the formulated model is equal to $(4+ j \times n_0)$, where j is the number of different size CM fractions, and n_0 is the arbitrarily chosen number of terms required for the approximation of the infinite series. Considering this, in order to minimize the computational efforts and to overcome some of the limitations present in the software used, it was initially decided to treat CM as a uniform material of one size, equal to the mass average radius, R_{avg}.

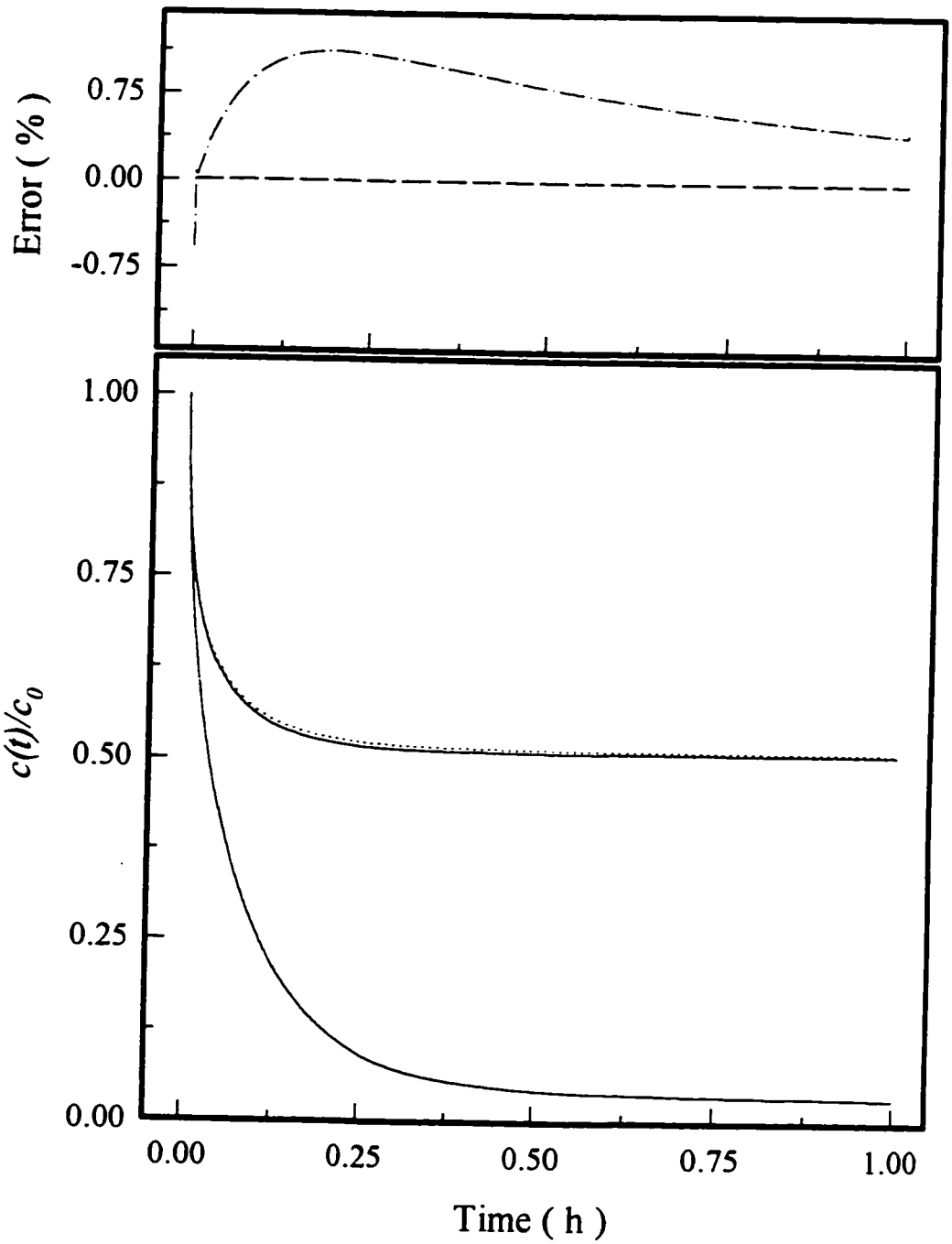


Figure 9-23. Comparison of the analytical and numerical solutions for the extraction of a solute from a spherical solid into the finite volume of the bulk liquid; and into the infinite volume of the bulk liquid. (A) - calculated concentration profile in the solute: (———) analytical; numerical (------); (B) - the relative error (%) of the numerical solution: *case 1* (–·–·–); *case 2* (———).

$$R_{avg} = \sum_{i=1}^{J} x_i R_i \tag{9.31}$$

Using the data in Table 9-1, R_{avg} was calculated to be 251 μm. This diameter was used in the preliminary tests to determine whether the model, in the proposed form, was able to describe the experimental results. It was found that, for the most part, the model predicted the patterns observed in the respective experiments. However, although the predictions for the initial stages of the process were in almost perfect agreement with the experimental results, the model failed to adequately describe the decrease in the SAE content for low SAE concentration in the meal. In this case, the model prediction showed a faster decrease in the SAE content than the one observed in the experiments (graphs in the following section). This led to the conclusion that the treatment of the meal as a uniformly sized commodity was an oversimplification, and that the meal particles with larger diameters contributed to the measured SAE content to a greater extent than initially expected. The phenomenon of the decrease in the rate of SAE extraction at low concentrations, occurring as a result of the lower rate of intraparticle diffusion (concentration of the SAE in the bulk phase was zero as shown in section 9.2), could have also been caused by a lower value of the effective diffusivity, D_{eff}. This made sense since the rate of intraparticle diffusion is proportional to the term D_{eff}/R^2, and since it is well known that the products of biological origin have variable diffusivities, the observed results could be explained by the dependence of the effective diffusivity on the SAE concentration. The concentration-dependent diffusivity gives rise to the non-linearity of the extraction problems, the solution of which is highly specific and requires additional computational efforts. Therefore, since the minimization of the computational efforts was one of the goals for the proposed mathematical description of the considered problem, the following method was proposed to account for the change in the extraction rate at low concentrations of the SAE in CM.

In this new approach, the commercial canola meal was considered as a mixture of particles with the same diameter but different effective diffusivities, $D_{eff,1}$ and $D_{eff,2}$. Thus, the lower rate of SAE extraction from the meal at low SAE concentrations would be described by the extraction from the fraction of CM with a lower effective diffusivity. The accuracy of the proposed method would therefore depend on the values of the diffusivities and the sizes of the respective fractions.

These values can be found using the data obtained in the experiments in which the enzymatic reaction was relatively fast, and therefore, it is reasonable to assume that the rate of the overall process was governed by the extraction step, *i.e*, intraparticle diffusion. To minimize the effect of the finite rate of reaction, only the data from the latter stages of the process should be considered.

When the above procedure was applied in this work, it was found that the experimental data obtained during the treatment of CM, using the highest enzyme concentration tested, were well described by the model when the meal was considered as a mixture of particles with a diameter of 502 μm, among which 94.6% had the effective diffusivity $D_{eff.1} = 1.738\times10^{-6}$ cm^2/s, and the rest $D_{eff.2} = 3.273\times10^{-9}$ cm^2/s.

It should be stressed that from the mathematical point of view a treatment of the meal as a mixture of particles with the same diameter but different effective diffusivities is not different from the one in which the particles in each fraction have the same effective diffusivity but different diameters. Using the already mentioned proportionality factor D_{eff}/R^2, it can be calculated that the diameter of the particles for which the rate of the intraparticle diffusion is the same as the one obtained using $D_{eff.2}$ is 3.66 mm. However, considering that particles with such a size are virtually absent in the CM (Table 8-1), the approach based on the concept of one diameter particles with two effective diffusivities was chosen for the final form of the model. This choice was also supported by the magnitudes of the effective diffusivities, $D_{eff.1}$ and $D_{eff.2}$, which fell into the range of diffusivities characteristic for the intraparticle and intrapore diffusions, respectively, observed during the sorption of heavy metals by biomaterials [Bhattacharaya and Venkobachar, 1984].

9.6.3 Estimation of the model parameters

The estimation of the model parameters was performed using a least-squares technique, namely Powell's algorithm, coupled with the ODE solver. In the first step, the system with no enzymatic reaction was considered, and the estimation was done using data regarding the extraction of SAE from the meal at three meal concentrations: 6.33%, 10.0%, and 20.0% w/v. Using this approach, the values of the partition coefficient can be obtained. Three types of the partition coefficient were considered. These were based on three types of sorption isotherms, namely: linear, Langmuir and Freundlich. The formulas for the respective coefficients are given in

Table 9-8. Parameter estimates for the extraction process based on the Langmuir and Linear sorption isotherms.

Parameter	Model	
	Langmuir	Linear
K_{Lg} (g/mL)	0.03879	n/a
k_{lg} (g/g$_{CM}$)	0.00348	n/a
k_{ads} (mL/g$_{CM}$)	n/a	8.34
MSC	5.85	5.52

n/a - not aplicable

Appendix B (Table B-1). The results obtained for the concentration dependent (Langmuir isotherm) and concentration independent (linear isotherm) partition coefficients are presented in Figure 9-24. The least-squares estimates for the respective constants in each of the considered sorption isotherms are given in Table 9-8. The attempts to use the partition coefficient based on the Freundlich isotherm were not successful. The results presented in Figure 9-24 showed that the extraction of the SAE from the CM can be described by both types of the partition coefficient. However, the Model Selection Criterion (MSC), which is useful for the discriminating of rival models [Micromath, 1994], showed (Table 9-8) that the model based on the Langmuir isotherm was more appropriate in describing the experimental data. In addition, when the simulation runs with the least squares estimates (Table 9-8) were performed to generate the results representing the experimental data presented in Figure 9-8, it was found that the better predictions were obtained using the partition coefficient based on the Langmuir isotherm.

Having determined the type of partition coefficient for the process of the enzymatic decrease in the SAE content in CM, the remaining parameters in the formulated model were estimated using the experimental data obtained from the experiments in which the active enzyme was used. The least square estimates obtained for each run considered are presented in Table 9-9. After preliminary estimation runs, the oxygen mass transfer coefficient k_La was set constant at 100 h^{-1}. Similarly, the stoichiometry of the enzymatic transformation of the SAE was kept equal 4. For some runs, not all of the data were used for the estimation. In the case of data from the experiments regarding the effect of oxygen concentration, only the run carried out at oxygen saturation

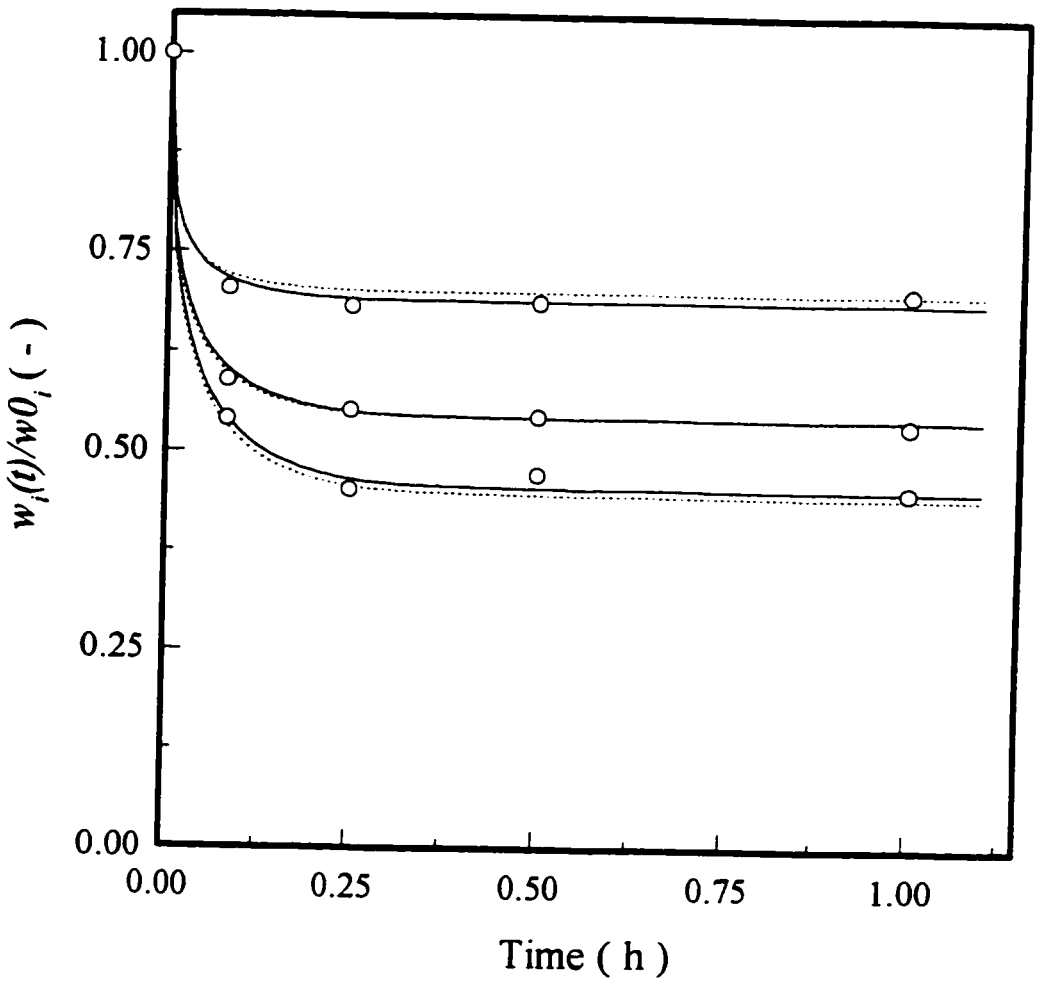


Figure 9-24. Changes in the SAE concentration during the extraction of the meal with a finite volume of the buffer-deactivated enzyme solution at different meal concentrations (w/v): 6.6%; 10.0%; 20.0%. Lines represent the predicted data using the least-square estimates from Table 9-8: (——) concentration independent; (——) concentration dependent. $w_i(t)$ -concentration in the CM after time *(t)* of the process; $w0_i$ - initial concentration of SAE

Table 9-9. Parameter estimates for the model describing canola meal system.[a]

Run #	Temperature [K]	Concentration: Enzyme [nkat/mL]	Meal [% w/v]	Oxygen[b] [-]	Parameter: $k_{5,CM}$ [h^{-1}]	$K_{i,O2,CM}$ [mol/mL]	$K_{O2,CM}$ [mol/mL]	K_{SAE} [mol/mL]	K_{PCM} [mol/mL]
1	323.2	4.0	10.0	0.21	0.0395	$1.664\ 10^{-6}$	$3.961\ 10^{-7}$	$5.535\ 10^{-5}$	$2.216\ 10^{-4}$
2	323.2	2.0	10.0	0.21	0.0304	$2.095\ 10^{-6}$	$1.169\ 10^{-7}$	$6.541\ 10^{-5}$	$5.733\ 10^{5}$
3	323.2	0.4	10.0	0.21	0.0304	$2.095\ 10^{-6}$	$1.169\ 10^{-7}$	$6.541\ 10^{-5}$	$5.733\ 10^{5}$
4	323.2	0.2	10.0	0.21	0.0152	$2.105\ 10^{-6}$	$1.078\ 10^{-19}$	$6.616\ 10^{-6}$	$2.686\ 10^{-5}$
5[c]	323.2	4.0; 2.0; 0.4; 0.2	10.0	0.21	0.1365	$2.561\ 10^{-6}$	$1.671\ 10^{-21}$	$7.708\ 10^{-6}$	$1.178\ 10^{-5}$
6	323.2	0.2	10.0	0.0	n/c	n/c	n/c	n/c	n/c
7	323.2	0.2	10.0	0.13	n/c	n/c	n/c	n/c	n/c
8	323.2	0.2	10.0	0.53	n/c	n/c	n/c	n/c	n/c
9	323.2	0.2	10.0	0.70	n/c	n/c	n/c	n/c	n/c
10	323.2	0.2	10.0	1.0	0.0106	$1.744\ 10^{-6}$	0.0	$9.191\ 10^{-5}$	$1.089\ 10^{-4}$
11	298.2	0.4	10.0	0.21	0.0122	$2.613\ 10^{-6}$	$1.991\ 10^{-14}$	$1.484\ 10^{-4}$	$6.575\ 10^{-5}$
12	308.2	0.4	10.0	0.21	n/c	n/c	n/c	n/c	n/c
13	345.2	0.4	10.0	0.21	n/c	n/c	n/c	n/c	n/c

General set of parameters[d,e]

	$k_{5,CM}$ [h^{-1}]	$k_{2,SAE}$ [mL/(mol h)]	$k_{3,PCM}$ [mL/(mol h)]	$k_{1,CM}$ [mL/(mol h)]	$k_{-1,CM}$ [h^{-1}]
$k_{i,0}$	$7.65\ 10^{8}$	$1.90\ 10^{22}$	$3.07\ 10^{-19}$	$9.67\ 10^{61}$	$2.01\ 10^{71}$
$E_{i,a}$	41.753	93.002	-158.247	241.753	334.005

[a] - using partition coefficient based on the Langmuir sorption isotherm (Table 9-8);
[b] - expressed in volume fraction of oxygen in the gas phase;
[c] - parameters describing degree of enzyme saturation: p_{2f}= 13.183; p_{3f} = 0.3677;
[d] - energies of activation, $E_{i,a}$, in kJ/mol;

[e] - the rate constants are given by:

$$k_{2,SAE} = k_{5,CM}/K_{SAE}\ ;\ k_{3,PCM} = k_{5,CM}/K_{PCM}\ ;\ k_{1,CM} = k_{5,CM}/K_{O2,CM}\ ;\ k_{-1,CM} = k_{1,CM}\,K_{i,O2,CM}\ ;$$

n/c - not considered for estimations.

condition was used for parameter estimation. The other runs were not considered since an experimental error associated with the procedure applied in this experiment was relatively big. The general set of parameters that would describe the process at any set of conditions was also considered in this work. Such a set was obtained by using the data from all the experiments focusing on the effect of enzyme concentration, and these pertaining to the effect of temperature.

9.6.3.1 Effect of enzyme concentration

The results obtained from the experiments focusing on the dynamics of SAE decrease at various enzyme concentration are shown in Figure 9-25. They were used to obtain the least square estimates of the parameters in the proposed model. At first, each run was considered separately, and the model predictions obtained with the respective least square estimates from Table 9-9 are shown as solid lines (Figure 9-25). A very good agreement was observed. However, in order to obtain a more general form of the model, the degree of system saturation with enzyme (Eq.(9.3)) was added to the rate equations and a new set of model parameters was estimated using all the data pertaining to the effect of enzyme concentration. The model predictions obtained with this set of parameters are shown in Figure 9-25 as dotted lines. Although the fit was not as good as the one when the runs were considered separately, the model still predicted the patterns observed in the experiments.

9.6.3.2 Effect of temperature

Considering that the general form of the model should also account for the effect of temperature on the process, the effect of this variable was also introduced into the model. Changes in the effective diffusivities with temperature were described using the equation of Wilke-Chang [Wilke and Chang, 1955], taking the diffusivities at 50°C as the reference:

$$D_{eff,T_2} = D_{eff,T_1} \frac{\mu_1}{\mu_2} \frac{T_2}{T_1} \tag{9-32}$$

where: μ_1 and μ_2 are the dynamic viscosities (cP) of the bulk liquid at temperatures T_1 and T_2 respectively.

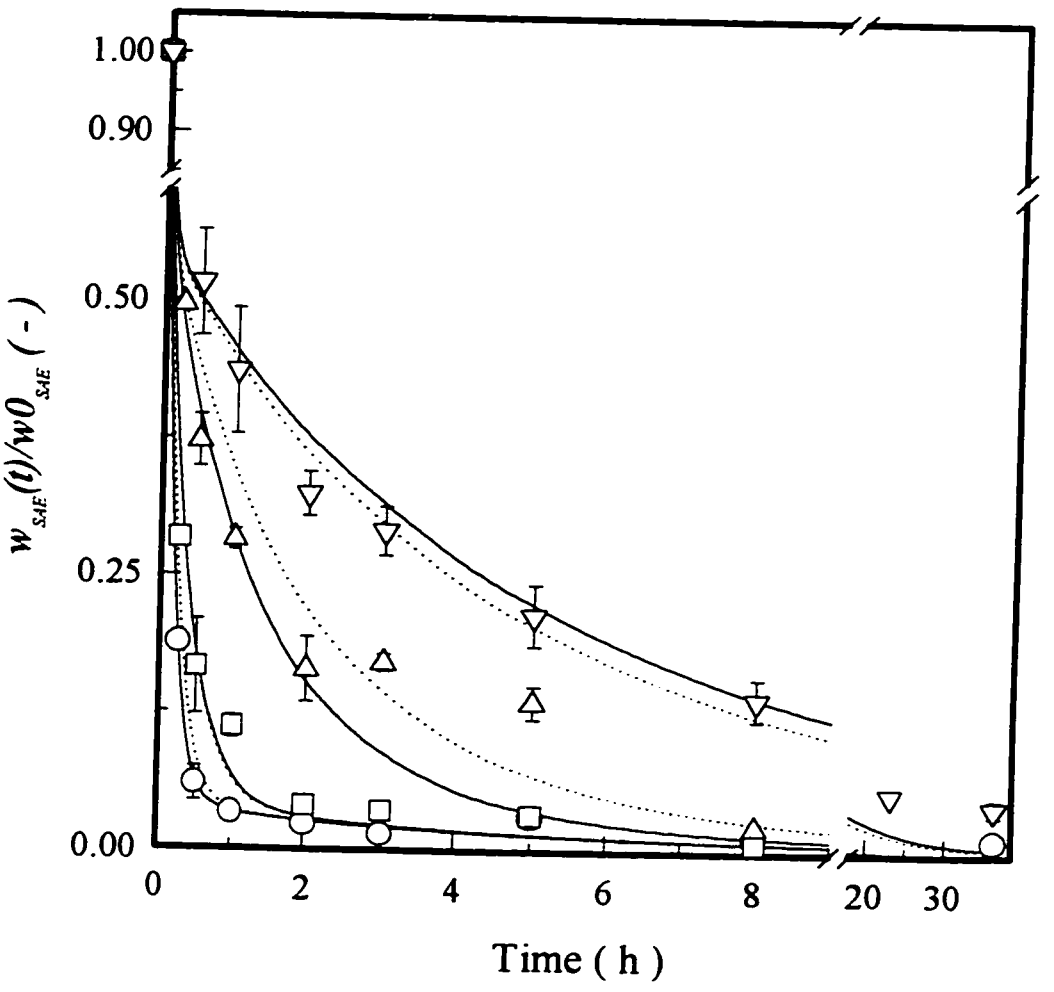


Figure 9-25. Effect of enzyme concentration on the dynamics of SAE decrease in 10.0% w/v CM suspension at 50°C: (○) 40 nkat/g; (□) 20 nkat/g; (Δ) 4 nkat/g; (▽) 2 nkat/g. Solid line predicted using individual set of parameters; dotted lines as obtained with the general set of parameters.

The changes in the viscosity of water (*i.e.*, neglecting the effect of proteins, and other water soluble compounds on the viscosity of the bulk liquid) were introduced into Eq.(9-32) through an empirical equation obtained from the linear regression on the data for water viscosity at different temperatures taken from Bolz and Tuve [1973]. The enzyme's kinetic constants were expressed using the Arrhenius relationship. The effect of temperature on the oxygen concentration in the bulk liquid was expressed using Henry's coefficient, and the effect of temperature on the enzyme deactivation was introduced using the model discussed in section 9.5.4. For practical reasons, it was decided to assume that the degree of system saturation with the enzyme is independent of temperature, and that the effect of this process variable on the sorption isotherm can be neglected. In order to narrow the parameter space during the least square estimation, the respective kinetic constant were represented by Eq.(8.15) [McLean, 1985].

The median temperature was set at 50°C, reducing the number of parameters to be estimated by half, as was discussed in section 8.4.5. The parameter estimates obtained after fitting the model to the experimental data for 20°C, 35°C and 72°C are given in Table 9-9. These estimates were used to predict the effect of temperature on the process for SAE decrease in CM (Figure 9-26). The model predictions obtained agreed well with the trends observed in the experiments. The observed differences can be explained through some of the simplifications used to incorporate the effect of temperature on the process outcome, for example neglecting the temperature effect on the sorption isotherm.

9.6.3.3 Effect of oxygen concentration

The effect of the initial oxygen concentration was also considered in this part of the work. However, as already discussed, the experimental errors encountered in these tests were relatively large and therefore, only the results obtained from the system which was initially saturated with oxygen were considered. The least square estimates for this run are given in Table 9-9. They were different than those represented by the general set of parameters used to predict the effect of enzyme concentration and temperature on the SAE decrease. The model predictions obtained using both sets of parameters are shown in Figure 9-27 together with the experimental data. The results showed that model with the general set of parameters overestimated the decrease in SAE content

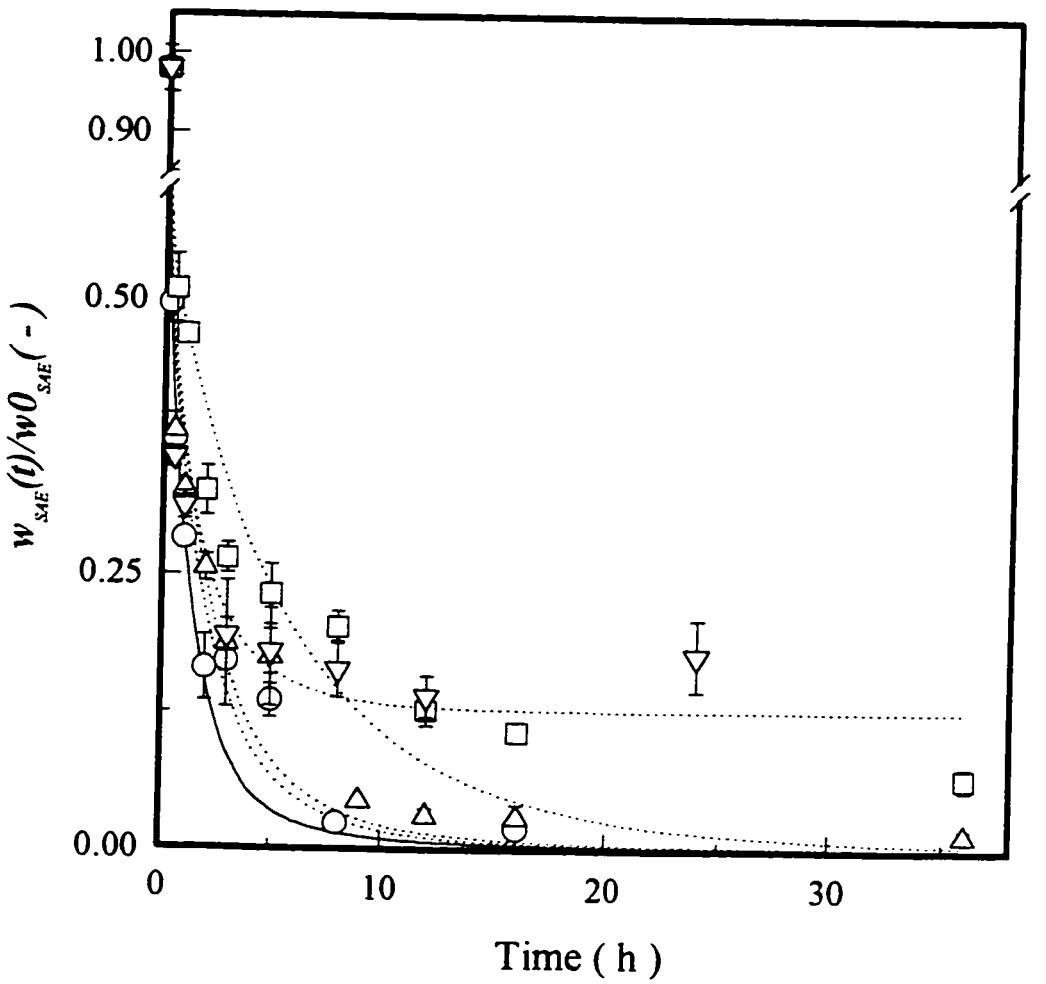


Figure 9-26. Effect of temperature on the dynamics of SAE decrease in 10.0% w/v CM suspension using enzyme at 4 nkat/g: (□) 20°C; (Δ) 35°C; (○) 50°C; (▽) 72°C. Solid line predicted using individual set of parameters; dotted lines as obtained with the general set of parameters.

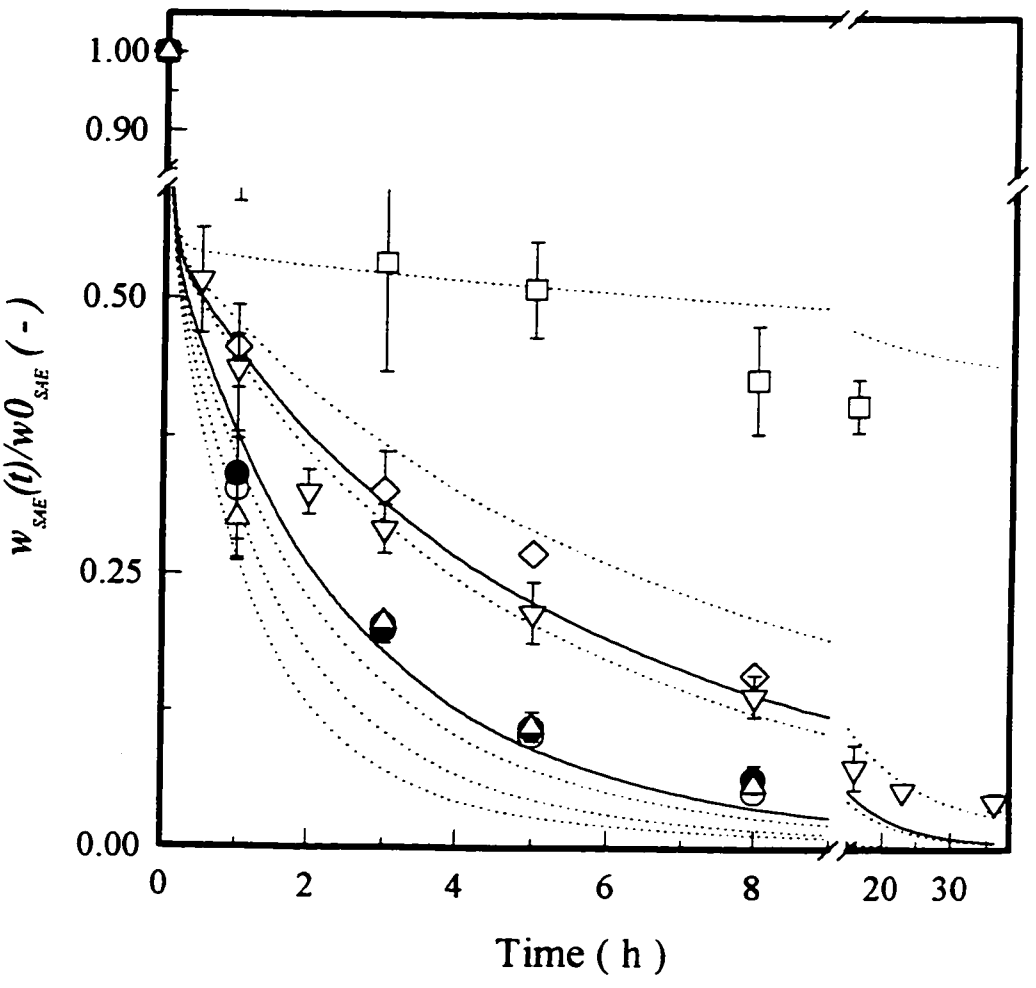


Figure 9-27. Effect of oxygen concentration on the dynamics of SAE decrease in 10.0% w/v CM suspension using 2 nkat/g of enzyme at 50°C and at the atmospheric pressure. Oxygen fraction in the gas phase: (●) 1.0; (Δ) 0.70; (○) 0.53; (▽) 0.21; (◊) 0.13; (□) 0.01. Solid lines represent model predictions using the individual set of parameters; dotted lines as obtained with the general set of parameters.

for oxygen concentrations higher than 53.0% (*i.e.*, when the gas phase contained less than 47.0% of nitrogen). In fact, the model was unable to account for the oxygen saturation conditions the presence of which can be seen from the experimental data (Figure 9-27). The increase in the oxygen concentration in the system from 53.0% to 100.0% did not result in any additional decrease in SAE. However, it should be noted that a very good agreement between the experimental results obtained at higher oxygen concentrations could have been observed when the model with the general set of parameters was used to simulate the process for the initial concentration of oxygen in the gas phase of 50.0%. The model described the experimental data reasonably well when the low oxygen concentrations were considered. The discrepancy between the model predictions, obtained with the general set of parameters, and the experimental results could be explained through the lack of specific information regarding the changes in the concentration of oxygen in the liquid and gas phases during the enzymatic process. Had these data been known, additional constraints on the parameters would have been introduced, thus leading to more realistic values of the least square estimates.

Considering the above, the model with the general set of parameters (Table 9-9) was used to perform some simulation of the investigated process.

9.6.4 Simulation results

The following is the discussion of some aspects of the enzymatic process based on the simulation results obtained with the general set of model parameters.

9.6.4.1 Dynamics of the overall enzymatic process

The formulated model described the changes in the concentrations of the SAE, oxygen, enzyme and product of the enzymatic reaction, P_{CM}, with time. Examples of these time-concentration curves are shown in Figure 9-28. Four limiting cases were considered: (A) the process is limited by the reaction step due to the low enzyme concentration; (B) process limited by the extraction of SAE from the meal; (C) process limited by the low oxygen concentration; (D) process not complete due to the thermal deactivation of the enzyme.

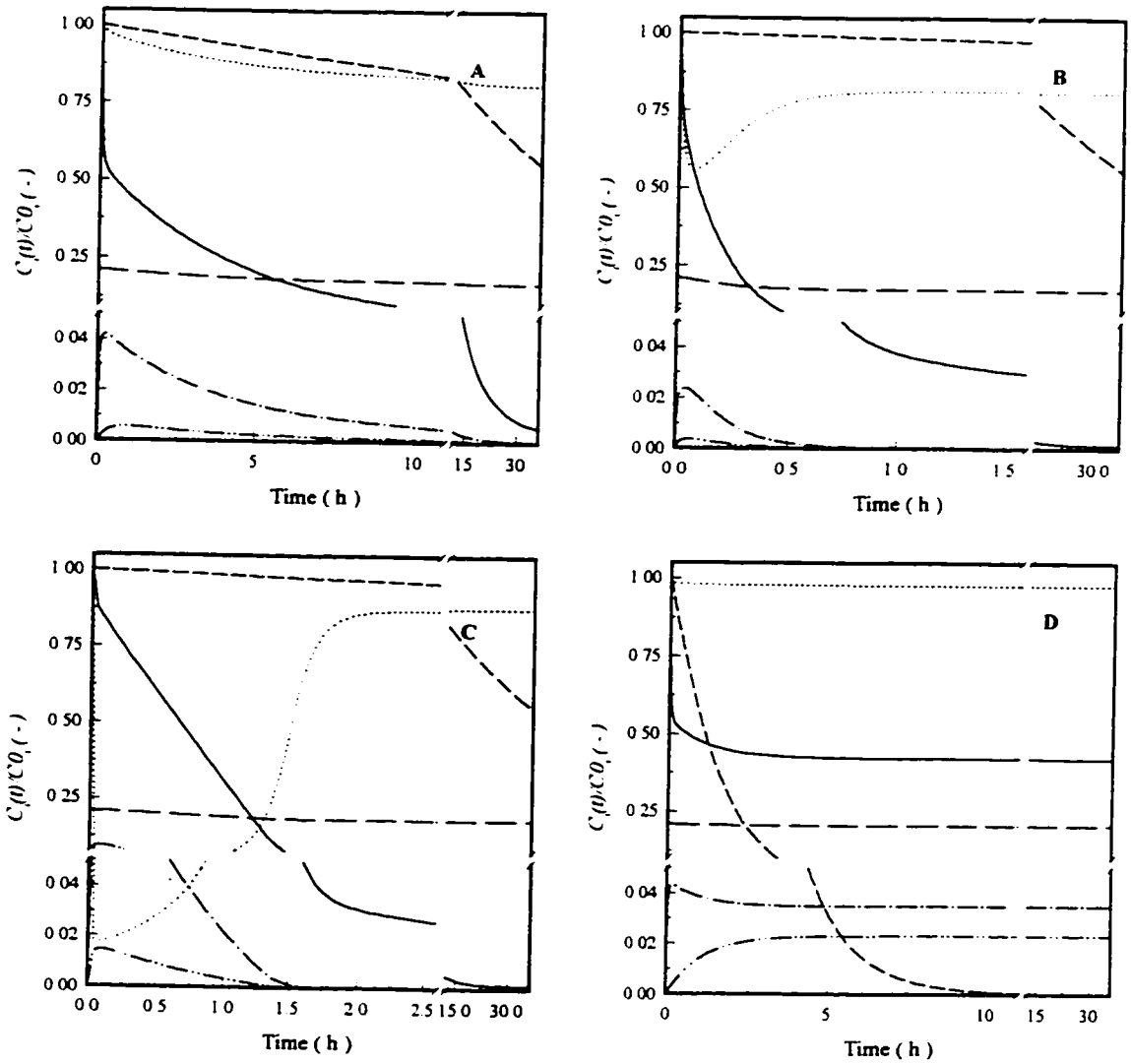


Figure 9-28. Dynamics of the enzymatic process to decrease SAE content in CM under four process conditions: (A) - enzyme concentration, E_{CM} = 0.1 nkat/mL, meal concentration = 10%, T = 50°C; (B) - E_{CM} = 2.0 nkat/mL, meal concentration 10.0%, T = 50 °C; (C) E_{CM} = 40 nkat/mL, meal concentration 100%, T = 50°C; (D) - enzyme concentration, E_{CM} = 0.1 nkat/mL, meal concentration = 10%, T = 80°C. All of the results were simulated with the general set of parameters assuming that the gas-phase was filled with air. (·····) - C_{O2l}; (———) w_{SAE}; (——) E_{CM}; (–·–·–) C_{SAE}; (–·····–) P_{CM}; (— — —) X_{O2}.

Considering *Case* A (Figure 9-28A), after the initial rapid decrease in phenolic content due to the extraction of SAE from the meal, the enzymatic process, became reaction limited, since the enzyme concentration was relatively low and did not provide sufficient means to decrease the SAE concentration in the bulk liquid to zero, thus decreasing the driving force for the process. In this case, the rate of oxygen transfer to the bulk liquid was at least as fast as the enzymatic reaction. The results presented simulated the process being carried out at 50°C and, therefore, enzyme deactivation did not play a significant role in the process outcome.

The results for *Case* B were generated using the same process conditions as for *Case* A, except that the enzyme concentration was increased 20 fold. In this case, the process became extraction limited after 30 minutes due to the fast enzymatic reaction that decreased the SAE concentration in the bulk to zero. Under these process conditions, an initial drop in the oxygen concentration in the bulk liquid was observed, which resulted in a decrease in the reaction rate. However, this decrease was not big enough to change the regime of the process, *i.e.*, it still became extraction limited since the enzyme concentration and oxygen concentrations were high enough to transform all the SAE extracted.

The opposite scenario was observed when the simulations were performed using the same process conditions as in *Case* B, but a 20 fold higher meal concentration. In this case, the process became reaction limited due to the low concentration of oxygen in the bulk liquid. Under these process conditions, the dissolved oxygen was almost instantly utilized, as shown by a drop in its level down to 10.0% of the initial concentration, within the first 5 minutes of the treatment. This resulted in the rate of the reaction (Figure 9-28C) being three times less than the rate observed in *Case* B, even though the enzyme concentration in the system was the same. The process became extraction-limited when the concentration of the SAE in the bulk liquid dropped to zero due to the enzymatic reaction.

The results presented in Figure 9-28D, (*Case* D) represent the outcome of the process carried out under the same conditions as in *Case* A, but at 80°C. In this case, the decrease in the SAE content was limited by the thermal deactivation of the enzyme. The rate of SAE decrease was governed by the rate of the enzymatic reaction, which after 2 hours, became slower than the rate of

the SAE diffusion from the meal. The process, when the enzyme was completely deactivated, reduced to the extraction of SAE into a limited volume.

9.6.4.2 Effect of meal and enzyme concentrations

Since the meal and enzyme concentrations influenced the process for decreasing the SAE content in the CM, it was decided to determine whether the proposed model could be useful in obtaining the optimum conditions for the process. In these tests, the effects of the meal and enzyme concentrations were considered. The results obtained were presented in the form of the differences between the results obtained in the enzyme system and those obtained when the meal was extracted with an infinite volume of the bulk liquid. Such a representation was considered since the ultimate result due to the presence of the enzymatic reaction in the bulk phase was a zero concentration of the extracted SAE, which is the same condition as the one observed when the extraction is carried out into an infinite volume. For simulation purposes, two systems were considered. In the first system, the concentration of the enzyme was kept constant in the liquid phase, while the other system represented situation where the concentration of the enzyme decreased with the decrease in the meal's concentration. The results obtained are shown in Figure 9-29. In the systems with a constant enzyme concentration in the continuous phase (dotted lines) the expected trend was observed: the lower the meal concentration, the smaller the differences in the extent of SAE decrease between the infinite and finite-volume systems. A similar effect was observed in the systems with the constant amount of the enzyme per unit mass of the treated meal, but only at the initial stages of the process. This pattern reversed as the process entered the regime governed by the rate of the enzymatic reaction. For that reason, in the cases of higher meal concentrations (*i.e.*, the enzyme concentration in the continuous phase also higher) the significant differences observed during the initial stages of the process were caused by the less efficient, partition coefficient governed extraction. On the other hand, in the latter phase of the treatment, the enzymatic reaction started to have a significant effect, causing a faster rate of SAE decrease in the continuous phase, thus bringing the conditions closer to those existing during the extraction of the SAE into an infinite volume of solution.

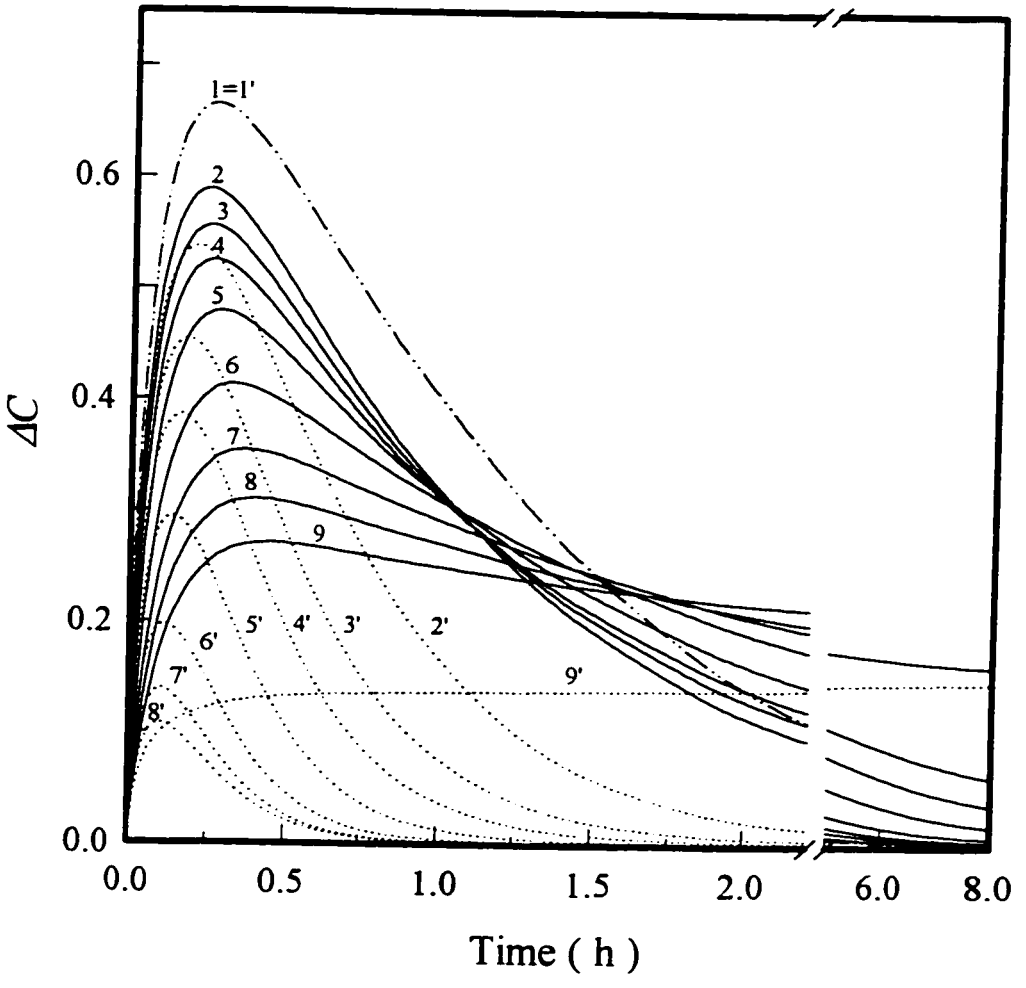


Figure 9-29. Effect of the meal and enzyme concentration on the deviation of the enzymatic treatment from the extraction of the SAE into an infinite volume of extractant. System I: constant enzyme concentration of 4.0 nkat/mL of the continuous phase, dotted lines, primed numbers; System II constant amount of enzyme per unit mass of CM 4 nkat/g, solid lines. Meal concentrations w/v: (1) 100%; (2) 50%; (3) 33%; (4) 25%; (5) 16.7%; (6) 10%; (7) 6.7%; (8) 5%; (9) 3.8%.

9.6.4.3 Effect of the delay in enzyme addition

Considering that the rate of the enzymatic transformation of the SAE depends on its concentration, it was of interest to determine whether a delay in the enzyme addition to the meal-buffer slurry could bring an increase in the overall rate of the process. The initial conditions of the process, at the moment of the enzyme addition, were obtained from the simulation results of the extraction of the meal with the deactivated buffer. These conditions were then used to solve the system of ODEs describing the investigated process. The results obtained showed (**Error! Reference source not found.**) that the 15 minute delay in the enzyme addition did not cause any adverse effects on the treatment but also did not result in a shorter process. Similarly, a delay of 45 also minutes did not have any effect on decreasing of the processing time.

The results obtained from the simulation runs showed that the proposed model is useful in simulating the enzymatic process under different sets of conditions and can prove beneficial in the design of the experiments for investigating this new method. However, in order for the model to be considered an independent tool in the optimization of the investigated process, additional work focusing on adjusting the model parameters to account for the effect of oxygen concentration should be performed. The parameter estimates found in this study were obtained using only the data pertaining to the changes in the SAE concentration in the meal with time and, therefore, not carrying any information about the changes in the concentrations of the other compounds. This led to the arbitrarily chosen values of some of the parameters, and even though, the model predictions obtained using the final set of parameters agree well with the experimental results, it seems advisable to build an experimental setup in which most, if not all, of the variables could be measured.

9.7 Solubilization of cell wall material of canola meal

As shown in the previous sections, the investigated enzymatic process for SAE decrease is limited by the intraparticle diffusion of the phenolics from the meal. To reduce this effect, it is possible to alter the structure of the cell-wall of CM using polysaccharide-hydrolyzing enzymes. Changes in the cell-wall structure caused by treatments with this type of enzyme resulted in an

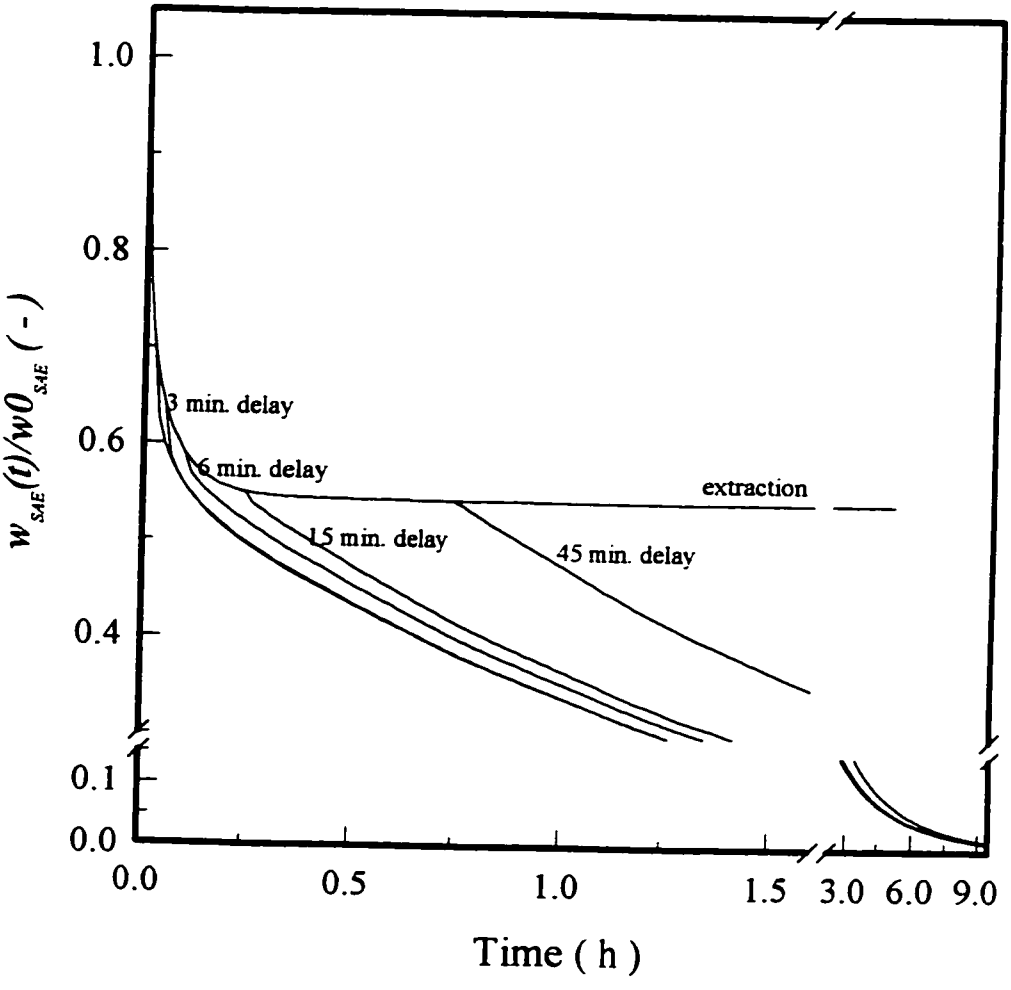


Figure 9-30. Effect of the delay in the enzyme addition on the SAE content decrease in CM at 50°C using an enzyme concentration of 4 nkat/mL.

increase in the extent of oil extraction from rapeseed seeds [Sosulski and Sosulski, 1990; Jensen *et al.*, 1990]. Gattinger *et al.* [1990] showed that commercial canola meal can be saccharified using an enzyme preparation secreted by *Trichoderma reesei* and two commercial carbohydrate-degrading enzymes preparations. According to their results, canola meal was saccharified mainly by hemicellulases, with the cellulases and xylanases being 18.0% and 35.0% less efficient, respectively. On the other hand, the cellulase preparation resulted in the highest concentration of the total water-soluble sugars released, indicating the greatest extent of solubilization of the cell-wall material. In this work two different enzymes preparations, xylanase and cellulase, that were never before tested with canola meal, were used to change the structural properties of CM particles.

9.7.1 Extent of the solubilization of the cell-wall material of CM

The extent of the solubilization of the cell-wall material of the meal was followed by measuring the changes in the concentration of total and free water-soluble sugars. The difference between these two concentrations gives the concentration of the polysaccharides fraction released from the meal.

In the initial experiments, the effect of the cellulase and xylanase concentration on the hydrolysis of the meal was examined. This was performed to assure that, during the treatment with a combination of polyphenol oxidase (PPO) and cell-wall solubilizing enzymes, the highest degree of solubilization was obtained. As shown in Figure 9-31, an increase in the cellulase and xylanase (GS35) concentration over 8.0 filter paper units (FPU) and 50.0 xylanase units (XU) per gram of the meal, respectively, did not have a significant effect on the release of water-soluble sugars after1 hour of treatment. In both cases about 14.0% of the meal was solubilized, as calculated based on the total carbohydrates content of the meal (Table 9-2). The xylanase produced a slightly higher degree of saccharification than cellulase. However, when non-diluted enzyme preparations were used, this effect was reversed (Table 9-10). These results showed that both enzyme preparations solubilized CM approximately to the same extent.

It was reported that autoclaving the suspension of canola meal was beneficial to the action of hemicelulases [Gattinger *et al.*, 1990], therefore, the effect of autoclaving as a meal

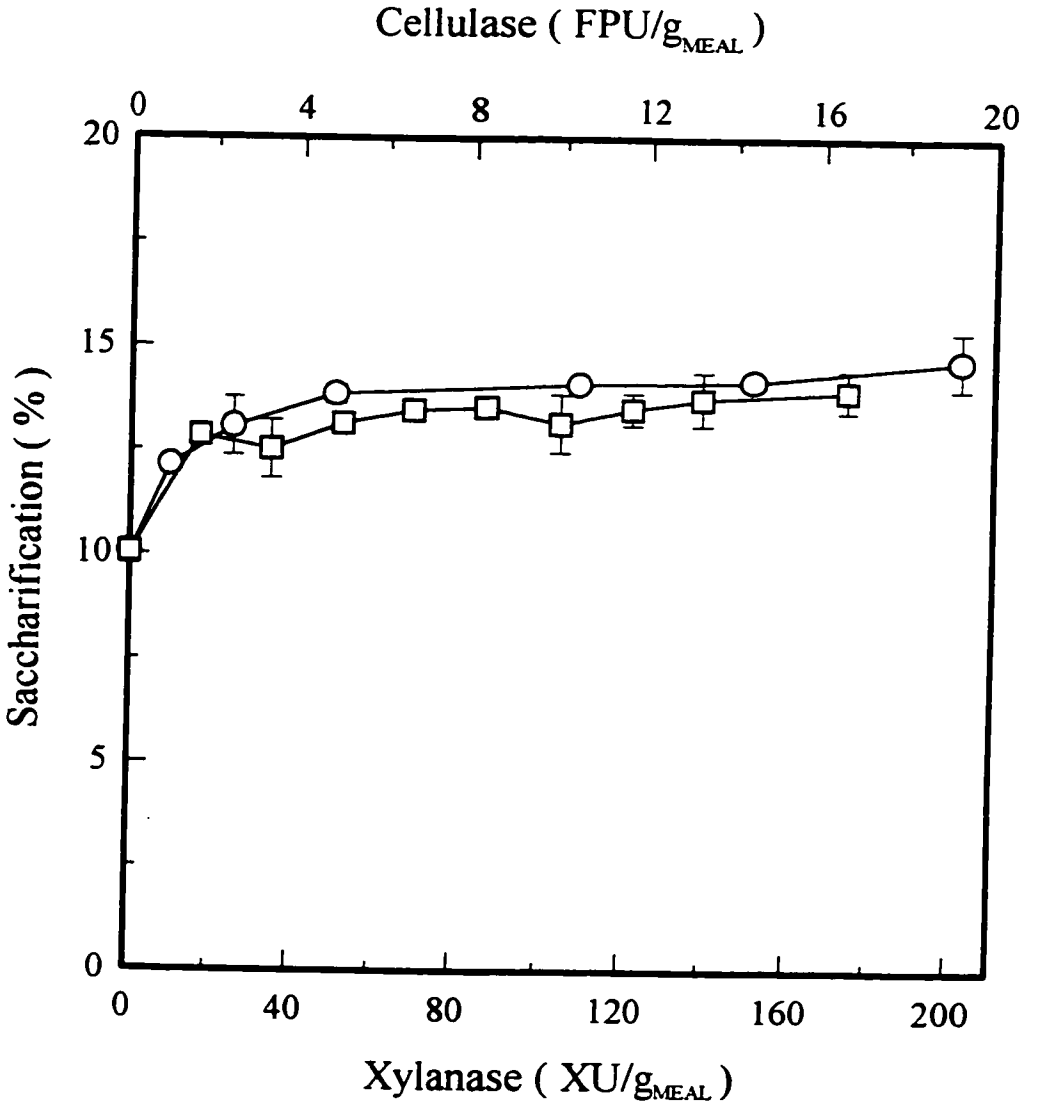


Figure 9-31. Effect of enzyme concentration on the extent of canola meal saccharification after 1 hour of processing: (O) - xylanase; (□) - cellulase.

Table 9-10 Effect of the meal pretreatment and the enzyme preparation on the extent of the hydrolysis of the cell-wall material of canola meal.

Meal Treatment	Enzyme Used	Water-soluble Sugars Released (% w/w)		Extent of cell-wall solubilization (%)
		Free	Total	
None	—	0.785	10.04	57.01
Autoclaved*	—	2.02	15.47	84.78
None	Xylanase	1.84	15.42	84.63
Autoclaved	Xylanase	3.30	16.78	92.09
None	Cellulase	4.14	15.64	85.84
None	PPO	7.11	10.42	57.19

* 30 minutes at 121°C.

pretreatment for cell-wall solubilization, was also considered in this work. The obtained results (Table 9-10) showed that this type of pretreatment was as efficient as a 1 hour-long treatment of the meal with cellulase and xylanase. It resulted in the saccharification of about 85.0% of the cell-wall material. The saccharification of the autoclaved meal by xylanase resulted in an additional release of free sugars (1.5% w/w of the meal) (Table 9-10).

The autoclaving of the meal suspension prior to the treatment with PPO and hydrolyzing enzymes was the most appealing for an eventual process to decrease the phenolic content in the pretreated meal with the altered cell-wall structure. However, from the nutritional point of view the autoclaving of the meal is unacceptable if the treated meal is to be used as a protein source since a large destruction (coagulation) of proteins is readily visible after this treatment. Due to this effect, such a pretreatment of the meal prior to its processing using PPO and the cell-wall solubilizing enzymes was not further investigated in this study.

The treatment of the meal only with the PPO from *T. versicolor* did not cause any additional solubilization of the meal (Table 9-10) but it did result in an increase in the concentration of free monosaccharides liberated from the meal. This observation showed that the PPO preparation used in this study included an enzyme capable of transforming water soluble di- and poly-saccharides, into monosaccharides.

The presence of this type of enzyme was confirmed in the model enzymatic system using sucrose as a substrate. Upon addition of the PPO preparation, the sucrose was transformed to glucose and fructose. This type of enzymatic reaction is characteristic for invertases.

9.7.2 SAE decrease in the solubilized meal

To evaluate the effect of the hydrolysis of the meal's cell-wall material on the extent of SAE content decrease, the meal was treated with either cellulase and PPO or xylanase and PPO. The results obtained with PPO alone served as a control.

The extent of the meal saccharification observed in these tests is shown in Figure 9-32. In the tests with cell-wall solubilizing enzymes, the solubilization of the meal took place mostly within the first hour of processing, and resulted in the saccharification of 85.0% of the meal's cell-wall material. The observed increase in the concentration of free sugars was caused by the presence of invertase in the PPO preparation. The effect of the meal treatment with xylanase and cellulase on the extent of the enzymatic decrease in SAE content of the meal is shown in Figure 9-33. The solubilization of the cell-wall material made it possible to completely eliminate the phenolics in the canola meal. The meals with no SAE were obtained after 16 and 36 hours of treatment with PPO in the presence of xylanase and cellulase, respectively. When none of these enzymes was used, the resulting meal still contained 2.0% of the initial amount of phenolics. It was noted that the initial rates of the enzymatic process decreased in the presence of xylanase and cellulase by 10.0% and 5.0%, respectively. This could have been caused by the high concentration of sucrose that was used as a stabilizer for the cellulase and xylanase preparations. However, when the rates of SA transformation were measured in the model enzymatic system in the presence of various concentrations of that sugar, no inhibitory effect was observed. Considering this, it has to be concluded that the observed inhibition was caused by some other compound present in the commercial enzymes preparations, *e.g.*, sodium benzoate.

The results obtained with the two enzymes capable of solubilizing cell-wall material confirmed that the rate limiting step for the enzymatic process to decrease the meal phenolic content in CM was the intraparticle diffusion of phenolics. This limitation can be overcome if 85.0% of the meal's cell-wall material is solubilized.

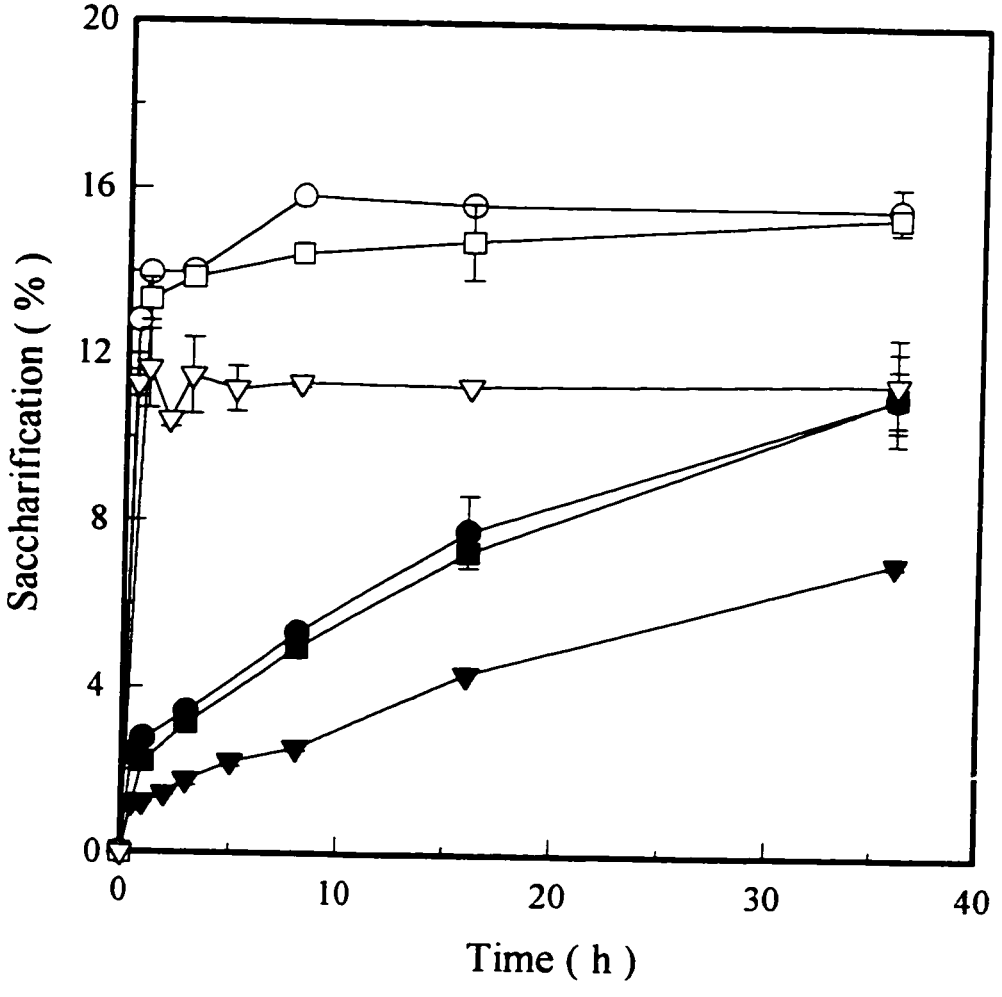


Figure 9-32. Solubilization of canola cell walls material at 50°C using 40 nkat/g of polyphenol oxidase (▽); in combination with cellulase 17 FPU/g (□), and xylanase 200 XU/g (○). Closed and open symbols represents free and total water-soluble sugars, respectively.

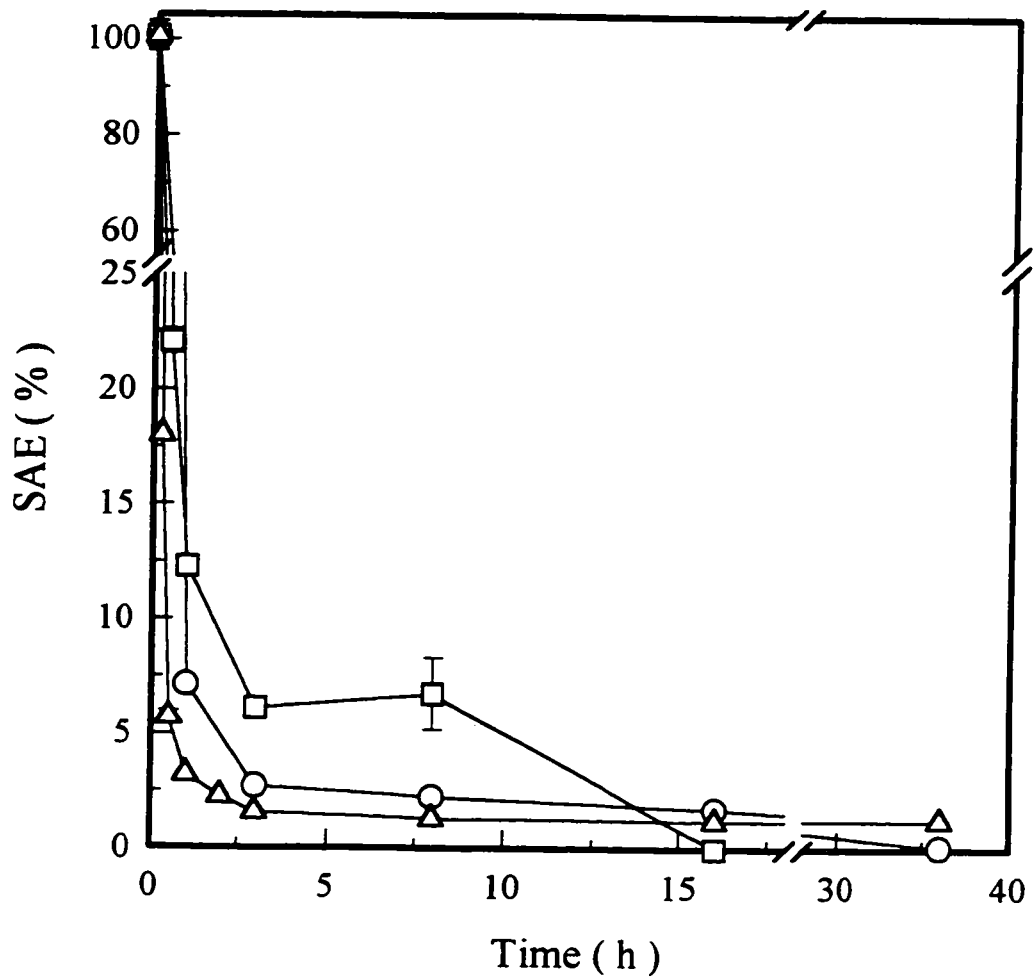


Figure 9-33. Effect of the meal's cell-wall solubilization on the decrease in SAE content of CM at 50°C using 40 nkat/g of PPO and: 17 FPU/g cellulase (O); 200 XU/g xylanase (□); no additional enzymes (Δ).

9.8 Enzymatic process carried out at low moisture content

The presence of the continuous phase, *i.e.*, the buffer and enzyme, in the basic system used to decrease the SAE content in the meal provided a means for a fast and efficient extraction of SAE from CM. This approach resulted in a meal with negligible phenolic content, as described in the previous sections. Unfortunately, the buffer-enzyme solution extracted 25.0% of the meal's proteins and more than 50.0% of the carbohydrates, therefore reducing the meal's nutritional value. To avoid these undesirable effects, the enzymatic process was carried out in a system with low moisture content. In this set of experiments, the extraction of proteins was eliminated because the amount of the enzyme-buffer solution used was smaller than the meal's water uptake. This means that the moisture content of the treated meal was not higher than 75.0% during the enzymatic treatment. Under such process conditions, the proteins are still extracted from the meal but, because of the imbibery properties of CM, they stay in the meal and, therefore, the meal's nutritional value is not affected.

9.8.1 Effect of moisture content

To determine the effectiveness of the proposed process modification, four moisture levels were investigated. The effect of enzyme concentration was also studied. All the tests were performed at 50°C to prevent a possible microbial contamination.

The obtained results showed (Figure 9-34) that in the systems where the enzyme concentration in the liquid was 5.0 nkat per mL, an increase in the moisture content from 45.0% to 75.0% resulted in a decrease in the SAE concentration by an additional 45.0%, producing a meal with an SAE content 83.0% lower than that of the untreated meal. This was expected, since the higher the moisture content, the better is the initial distribution of the enzyme and, furthermore, the longer is the period when the extraction of the SAE and their enzymatic transformation can take place.

Comparing the results obtained in the system with 65.0% moisture content and 6.75 nkat/mL to those obtained in the system with 75.0% moisture and 5 nkat/mL, shows that after a "saturation" of the system with water, an increase in the enzyme concentration had a larger effect (former case) on the SAE decrease than an increase in the moisture content.

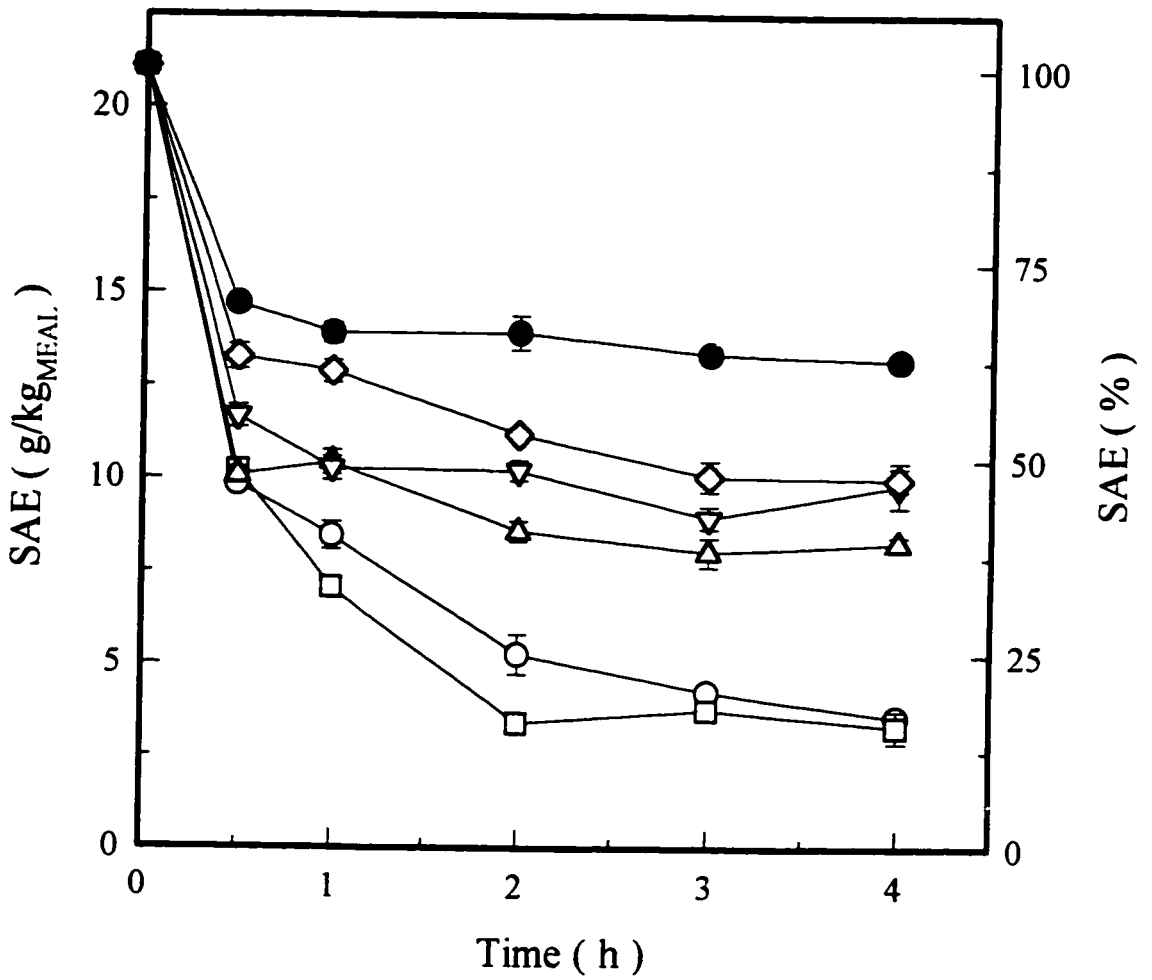


Figure 9-34. Effect of the moisture content and the enzyme concentration on the dynamics of SAE content decrease in canola meal at 50°C. Moisture content: 45% (●) 4.75 nkat/g or 5 nkat/mL; 45% (▽) 15 nkat/g or 15 nkat/mL; 55% (◊) 6.65 nkat/g or 5 nkat/mL; 55% (△) 15 nakt/g or 11.27 nakt/mL; 65% (□) 15 nkat/g or 6.75 nkat/mL; 75% (○) 15 nkat/g or 5 nkat/mL.

Thus, it can be said that both the enzyme concentration and the moisture content had some effect on the decrease of SAE content in canola meal, and their action was coupled. The systems with even a high enzyme concentration could not be effective at decreasing the SAE concentration if the moisture level was low, because of the restriction of the distribution of enzyme and the poor extraction of SAEs. Furthermore, in the systems with low moisture content the critical moisture level at which the enzyme can still be active was reached faster due to the water evaporation.

Considering the above it was decided, that all tests would be performed at an initial moisture content equivalent to the meal's water uptake.

9.8.2 Effect of additional moisture content

To determine the effects of the water evaporation on the enzymatic treatment during the process carried out at low moisture level, the meal was supplemented with additional moisture to compensate for the water evaporation. The tests were performed at 50°C, 70°C, 90°C and 110°C, though only results regarding the lowest and highest temperatures are presented since they represent the general trends observed. The results showed (Figure 9-35) that the evaporation of water at 50°C was relatively slow and, therefore, the additional water added during the treatment did not enhance the enzymatic process and resulted in the meal with a slightly higher SAE content. The reason for this adverse effect could be the decrease in the enzyme concentration in the aqueous phase. On the other hand, at 110°C, the supplementation of the system with additional water caused an extra 8.0% decrease in the SAE concentration in the treated meal. At this temperature, in the system not supplemented with moisture, the process of SAE content decrease stopped when the meal became dry because both the extraction of the remaining SAE and the activity of the enzyme were affected. The addition of extra water, brought back, to the some extent, the favourable condition for the enzymatic process. This result showed that the enzyme was not deactivated at 110°C during the first hour of the treatment.

9.8.3 Effect of temperature

The results presented in Figure 9-35 showed that the rate of the SAE decrease was higher at 110°C than at 50°C, despite the fact that the moisture content at the higher temperature

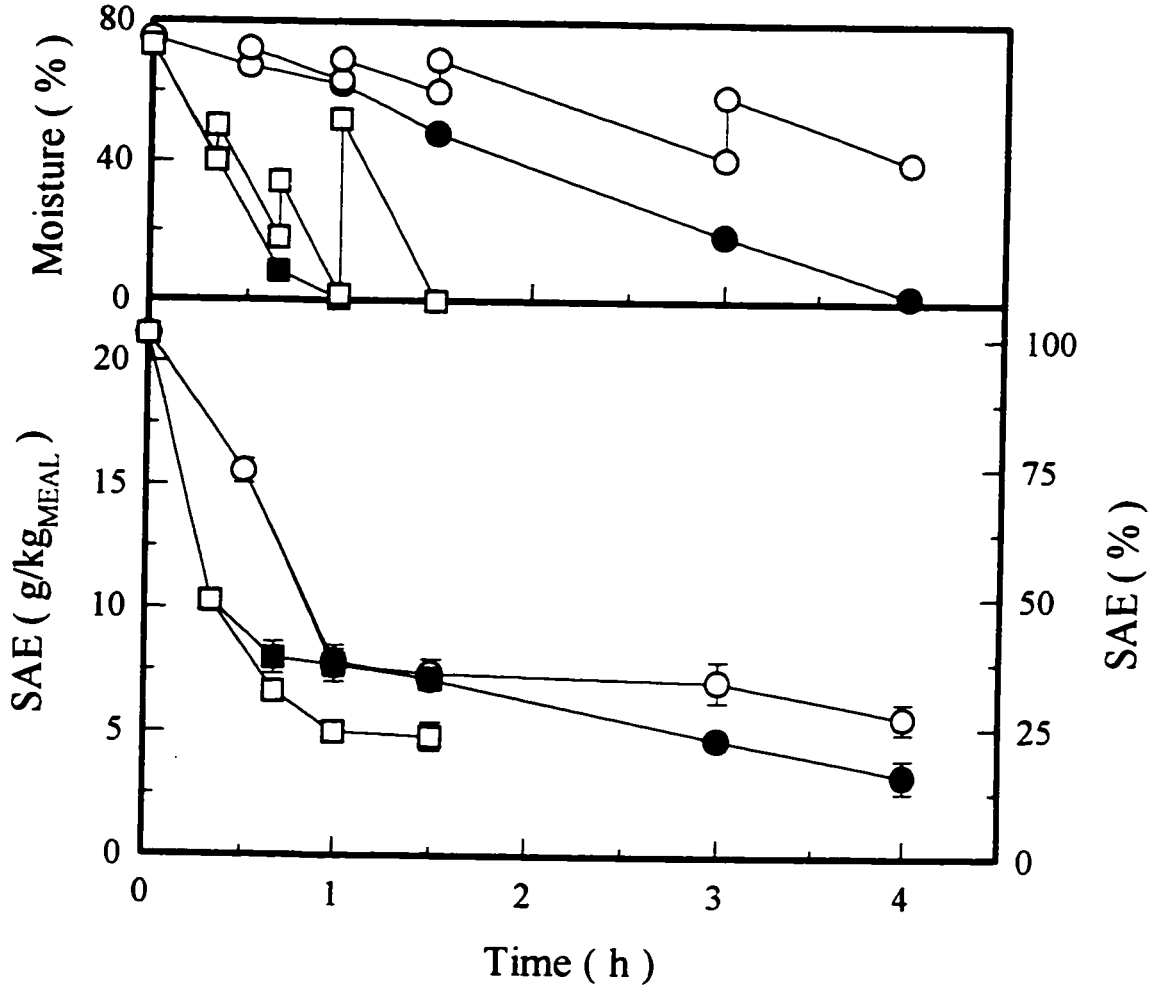


Figure 9-35. Dynamics of SAE decrease in canola meal at the initial moisture content of 73% and the enzyme concentration of 20.0 nkat/g with (open symbols) and without (closed symbols) moisture supplementation: (□) 110°C; (○) 50°C.

decreased more rapidly, causing less favorable conditions for the enzymatic process. Considering this, the additional tests were performed to evaluate the optimum temperature for the process carried out at the 75.0% initial moisture content. The results obtained at 15°C, 25°C, 50°C, 70°C, 90°C and 110°C (Figure 9-36) showed that the highest initial rate for SAE decrease was at 110°C. However, at this temperature the SAE content was not reduced completely, although the enzyme concentration was 5 fold higher than in the tests shown in Figure 9-35. Compared to 110°C, the processes carried out at the lower temperatures were slower but resulted in the meals with a lower SAE content.

One test was also carried out at a constant moisture level at 90°C in an autoclave at atmospheric pressure. In this case, the SAE content at the end of the test was higher than in the test that was carried out at the same temperature but with the initial moisture content of 75.0% (Figure 9-36). Most likely, the reason for this was a low concentration of oxygen due to the presence of the water vapor as the main component of the gas phase in the system. The importance of oxygen on the effectiveness of the enzymatic process was already demonstrated (section 9.5.5) but can also be seen from the results obtained at 15°C (Figure 9-36).The decrease in the SAE content was greater in the system that was sparged with air.

The results presented here showed that the enzymatic process to decrease the phenolic content in the meal can be performed at low moisture levels, which has a beneficial effect on the overall quality of the treated meal since valuable proteins and carbohydrates are not extracted.

9.9 Decrease in the SAE content in the presence of hexane

Considering that the industrial processing of canola seeds is based on the extraction of canola oil with hexane, it was of interest to investigate whether the oxidase from *T. versicolor* can cause a decrease in the phenolics content of the meal in the presence of hexane at a relatively low level of the moisture in the system.

9.9.1 Effect of the amount of aqueous phase

The effect of the meal's moisture level (aqueous phase) on the extent of SAE content decrease in the presence of hexane is shown in Figure 9-37. The results obtained showed a steady

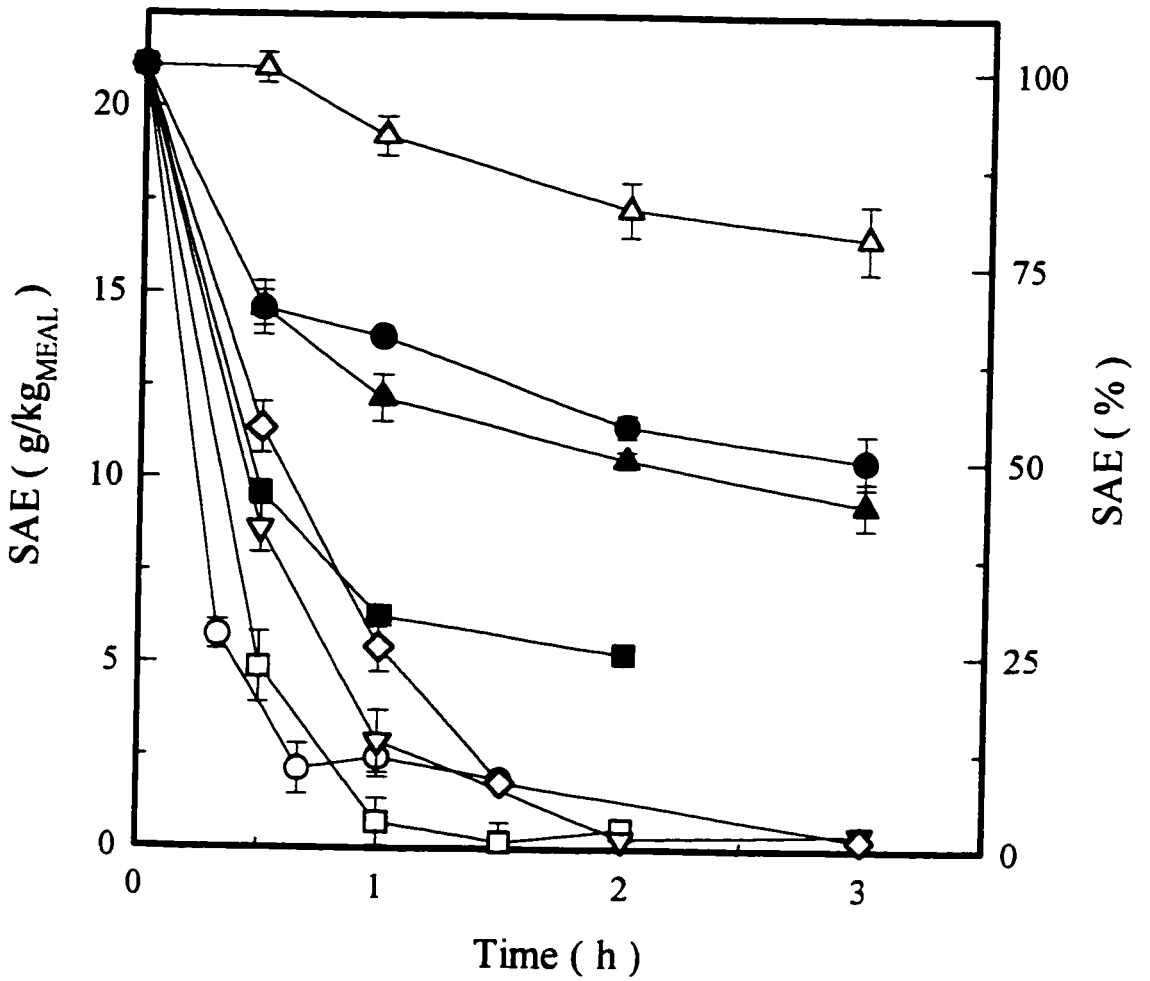


Figure 9-36. Effect of temperature and oxygen concentrations on the dynamics of SAE decrease in canola meal at the initial moisture content of 75% and the enzyme concentration of 100 nkat/g: (○) 110°C; (□) 90°C; (■) 90°C in the autoclave; (▽) 70°C; (◊) 50°C; (●) 25°C; (△) 15°C; (▲) 15°C with air.

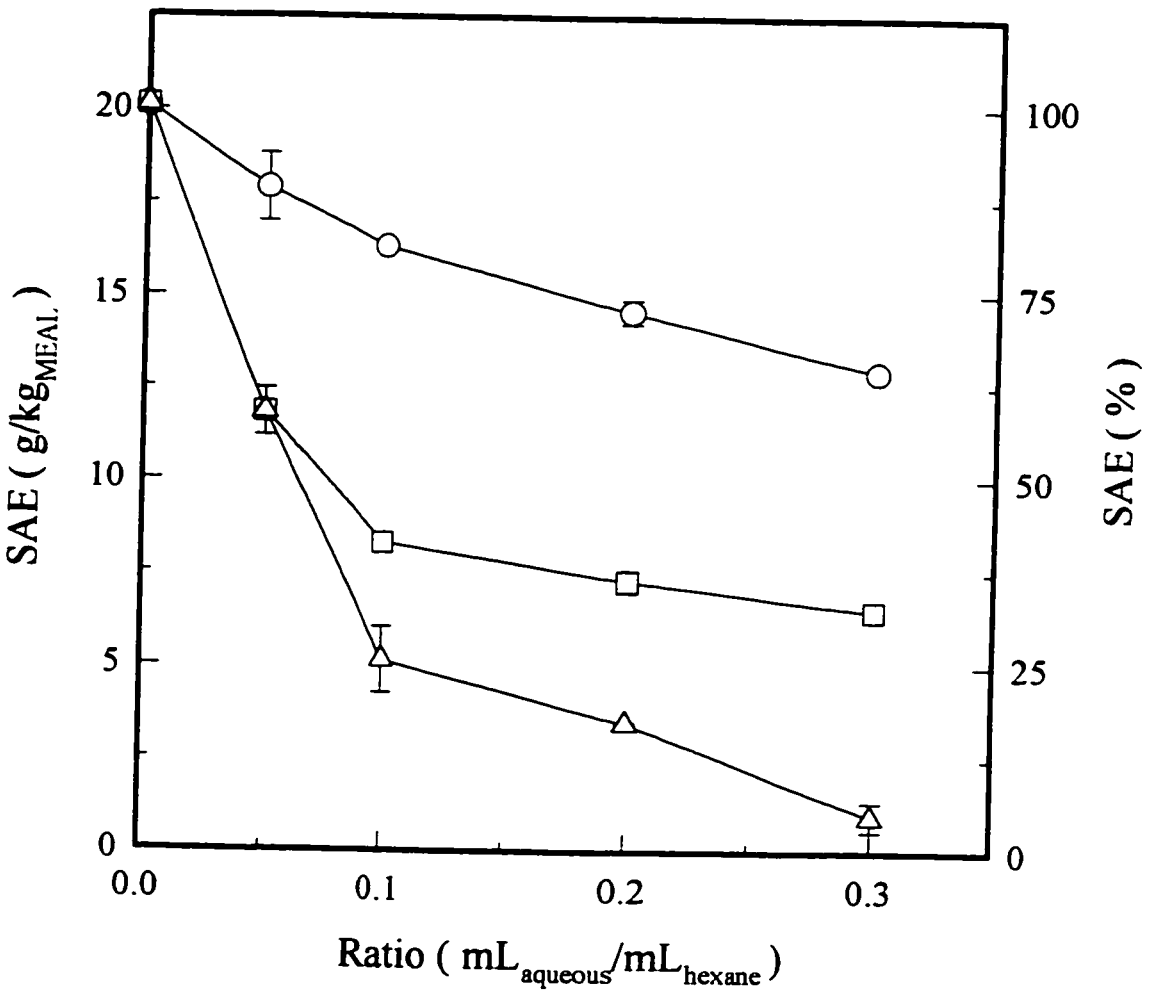


Figure 9-37. Effect of the volume of the aqueous phase on the extent of SAE content decrease in canola meal at the ratio of meal to hexane of 10 at 30°C after 1 hour of treatment. Deactivated enzyme (○); active enzyme: (□) constant enzyme to meal ratio of 20 nkat/g, (Δ) constant enzyme concentration in the aqueous phase of 40 nkat/mL.

decrease in the SAE concentration in the treated meal with an increase in the system moisture content from 30.0% to 75.0% in the case when the deactivated enzyme was used. These results showed that the meal's water uptake was lower in the presence of hexane, than when the treatment was carried out in the enzyme-buffer solutions (Figure 9-8). When the aqueous phase consisted of 40 nkat/mL of the enzyme preparation, the decrease in the SAE content varied from 45.0% to 95.0% for the systems with 30.0% and 75.% moisture content, respectively. The decrease in the concentration of the enzyme in the aqueous phase resulted in a lower degree of SAE degradation. The results obtained with the deactivated enzyme (Figure 9-37) showed that because of a lower meal water uptake encountered in the presence of hexane, the aqueous solution of the enzyme can move freely in the system and, therefore, the enzymatic process may be more effective than the one carried out at the same moisture content but in the absence of hexane (Figure 9-34).

9.9.2 Effect of temperature

To investigate the effect of temperature on the initial rate of SAE decrease in canola meal in the presence of hexane as the main component of the continuous phase, the process was carried out for 30 minutes at temperatures ranging from 10°C to 70°C. Two moisture and two enzyme concentrations were tested. The obtained results showed (Figure 9-38) that the optimum temperature for the enzymatic process was lower than when the treatment was carried out only in the aqueous phase (Figure 9-14). The temperature of 40°C was optimum for the enzyme concentration of 20.0 nkat per gram of the meal, at 75.0% moisture content. For the moisture level of 30.0% and the same amount of enzyme in the system, the optimum temperature was not as well defined but appeared to be around 40°C. However, in the system where the enzyme concentration was three times higher, the optimum temperature was shifted towards 30°C.

9.9.3 Dynamics of SAE decrease in the presence of hexane

To investigate the dynamics of the decrease in the SAE content in the presence of hexane, the process was carried out for 20 hours at three enzyme concentrations and two moisture levels. The results (Figure 9-39) showed that a 98.0% decrease in the SAE content was obtained after 20 hours of processing in the system where the concentration of enzyme was 20.0 nkat/g and the

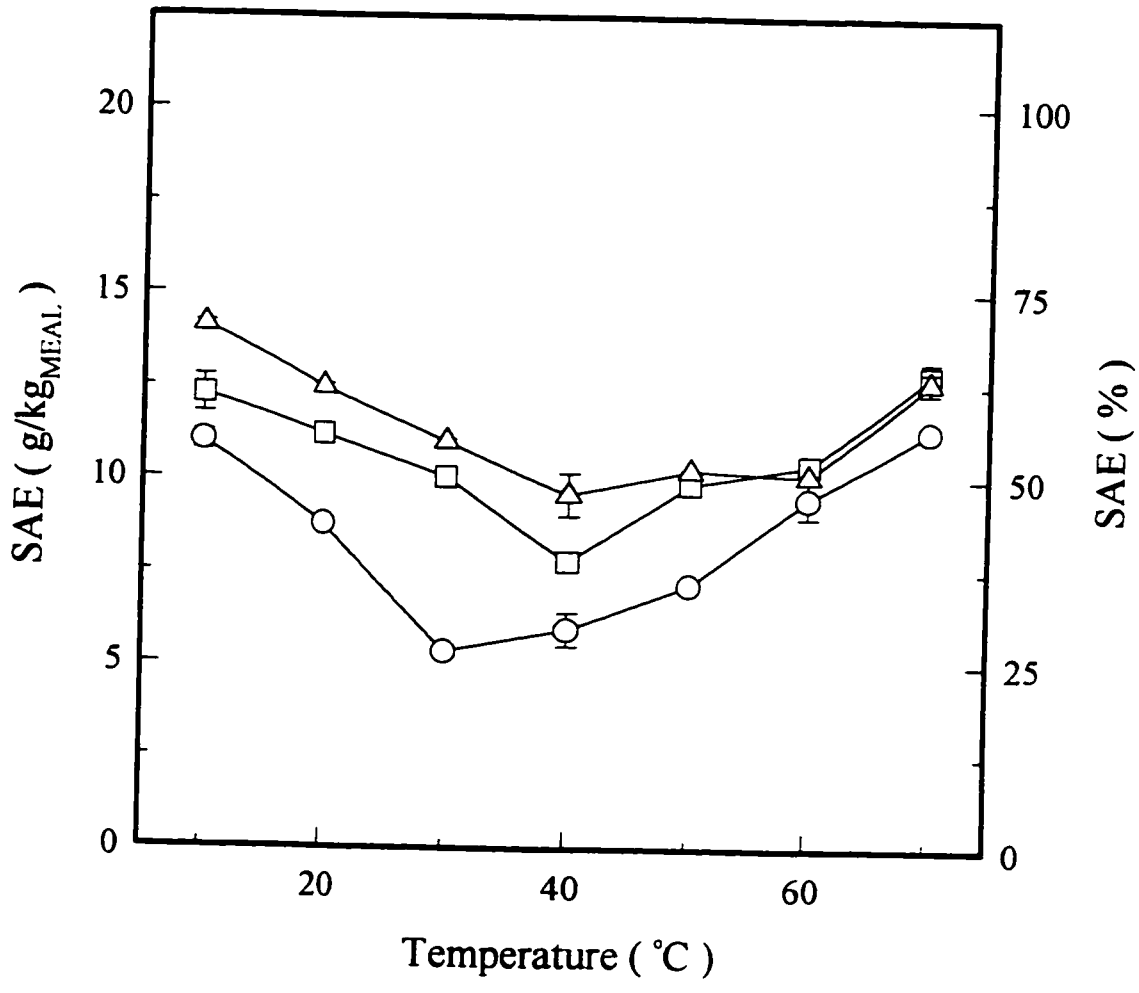


Figure 9-38. Effect of temperature on the extent of decrease in SAE content of CM in the presence of hexane after 30 minutes of treatment. Enzyme concentration: (Δ) 20 nkat/g/mL$_{aq}$, 30.0% moisture; (□) 20 nkat/g/3 mL$_{aq}$, 75.0% moisture; (○) 60 nkat/g/3mL$_{aq}$, 75.0% moisture.

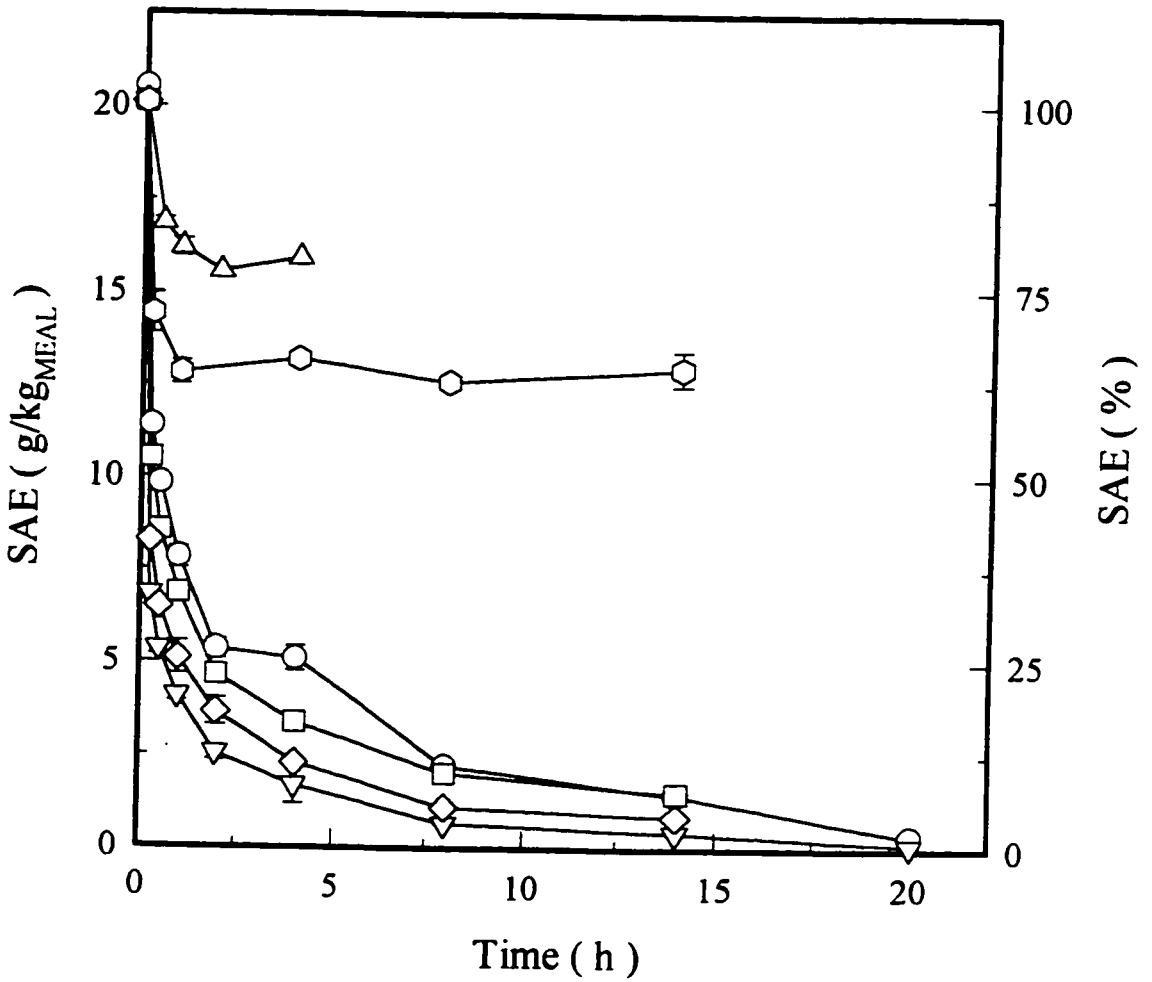


Figure 9-39. Dynamics of SAE content reduction in the presence of hexane at different working enzyme concentrations and moisture contents at 30°C. Enzyme concentration: (○) 20 nkat/g, 30.0% moisture; (□) 20.0 nkat/g, 75.0% moisture; (▽) 60.0 nkat/g, 75.0% moisture; (◇) 40.0 nkat/g, 75.0% moisture; (⬡) 0.0 nkat/g, 75.0% moisture; (△) 0.0 nkat/g, 30.0% moisture.

moisture level was 30.0%. After the same processing time, a meal with virtually no SAE was obtained when the moisture level and the enzyme concentration were 75.0% and 60.0 nkat/g, respectively. Decrease in the enzyme concentration caused a decrease in the rate of SAE content decrease. However, the lowest rate was observed in the system with 30.0% moisture content.

The results presented above showed that it would be possible to carry out the enzymatic decrease of SAE content in canola meal in the system with hexane as a main component of the continuous phase. This process would, however, require a sufficient volume of the aqueous phase, which may have a negative effect on the extraction of canola oil. Furthermore, the effect of the oil content on the efficiency of the enzymatic step should also be examined since the results obtained in preliminary experiments (data not shown) indicated that presence of canola oil reduced the effectiveness of the enzymatic method. The effect of the products from the enzymatic transformation of SAE on the quality of canola oil should also be addressed.

9.10 Comparison of the enzymatic with other methods for SAE content reduction

Comparison of the new enzymatic method with other methods that are used to decrease the SAE content in rapeseed meals was also performed in this work. In order to perform a direct comparison, the commercial canola meal was treated using the following four methods: lime treatment, autoclaving, boiling and MeOH-NH_3-hexane extraction. In some tests, each of the methods considered was combined with the enzymatic step to evaluate the possibility of obtaining a meal with a lower SAE content than after a single-method treatment. Solvent extraction of the meal with either ethanol or methanol was not considered because it was possible to extract all phenolics using organic solvents (section 9.3.1).

The results obtained showed that the enzymatic step was the most efficient among the treatments considered (Table 9-11), even though the optimum conditions for this process were not used. It resulted in an 85.0% decrease in the SAE content. This method was followed, in order, by the lime treatment, boiling, buffer extraction, and autoclaving. The MeOH-NH_3-hexane method was the least efficient, producing the meal with the phenolics content decreased by only 7.0%. The

Table 9-11. Comparison of four reported methods for phenolics content reduction with the proposed enzymatic treatment.*

Primary Meal Treatment	Additional Treatment	Residual SAE	
		[g/kg_{meal}]	[%]
Untreated		18.13 ± 0.53	100.00
Buffer (control)[a]	–	10.12 ± 0.10	55.27
	$Ca(OH)_2$	4.88 ± 0.55	26.64
	Autoclaving	8.75 ± 0.25	47.79
Enzyme[a]	–	2.79 ± 0.10	15.23
	$Ca(OH)_2$	1.93 ± 0.17	10.53
	Autoclaving	3.92 ± 0.14	21.42
MeOH-NH_3-Hexane[b]	–	17.01 ± 0.23	92.93
	Enzyme	2.42 ± 0.01	13.23
	Buffer	10.77 ± 0.20	58.80
Boiling[c]	–	8.51 ± 0.28	46.49
	Enzyme[d]	2.78 ± 0.62	15.19
	Buffer[d]	7.55 ± 0.17	41.23
Autoclaving[e]	–	14.83 ± 0.36	80.97
	Enzyme	5.45 ± 0.73	29.76
	Buffer	12.12 ± 0.81	66.19
$Ca(OH)_2$ [f]	–	4.97 ± 0.36	27.17
	Enzyme[h]	2.94 ± 0.16	16.04
	Buffer[g]	5.73 ± 0.49	31.27
	Buffer[h]	7.44 ± 0.30	40.65

* Results from enzymatic treatment not adjusted for product interference.

[a] The liquid phase/meal ratio of 5, enzyme concentration 20 nkat/g, pH of buffer 4.5, incubation at 30°C for 1 hour, meal separated by filtration and left undisturbed until dryness. Control prepared by replacing the enzyme preparation with water.

[b] Meal extracted with 5% ammonia in methanol at the ratio extractant/meal of 10 for 2 min. in the homogenizer. After quiescent period of 25 min. the hexane was added and extraction repeated. Meal separated by filtration with duplicate washing of residue with pure MeOH.

[c] Meal boiled in water for a period of 1 hour and separated by filtration and dried in the desiccator.

[d] The ratio liquid phase/meal had to be doubled because of the change in the properties of the meal after first treatment.

[e] Meal autoclaved for 30 minutes at 121°C and dried in the dessicator.

[f] Meal at 54% moisture level mixed with $Ca(OH)_2$ at the ratio 24/1. System was mixed occasionally during first 12 hours and then dried in dessicator.

[g] The pH of the buffer was 2.5, which gave the pH in the system of 5.3

[h] The pH of buffer was 4.5, which gave the pH in the system of 6.4.

low effectiveness of this method obtained in this work (Table 9-11), was probably caused by the mixing regime employed in the experimental procedure. In the original method the extraction of the meal with MeOH-NH_3-hexane was carried out in a Polytron, while in this work a high speed homogenizer was used. However, the lack of any effect associated with the washing step is somehow surprising, and remains unexplained.

When the lime treatment was applied to the enzyme-treated meal, an additional 5.0% decrease in the SAE content was observed. When the order of these two treatments was reversed, the meal contained 11.0% less SAE than after the lime treatment only, but was slightly higher than after a single enzymatic step.

Autoclaving had a less profound effect on the SAE content of the meal. A 6.0% increase in the SAE concentration was observed when this treatment was applied to the enzyme-treated meal. This increase could have been caused by the presence of additional phenolic compounds that were released during autoclaving. This kind of explanation is supported by the results obtained when the autoclaved meal was treated either with the enzyme, or with the buffer. In these tests the obtained meals had 14.0% and 11.0% higher SAE content than when these two methods were applied to non-autoclaved meal, even though the volume of the continuous phase was doubled (Table 9-11).

Treatment based on the boiling produced the meal with 10.0% lower SAE content than the buffer extraction alone, but was 31.0% less efficient than the enzymatic method. The buffer extraction of the boiled meal resulted in an additional 5.0% decrease in the SAE content. The boiling of the meal did not have any effect on the extent of enzymatic decrease in the SAE content.

The data shown in Table 9-11 were not adjusted for the presence of any reaction products that could interfere with the SAE measurements. However, the results obtained after the buffer and the enzyme treatments of the lime-processed meal suggested that such an interference could be present. The treatment with a more alkaline buffer should result in a higher degree of phenolics removal (extraction plus chemical reaction), and although, the results indicated the opposite pattern, these should be considered only as apparent ones, because the actual SAE concentration in the meal is lower. This confirms the existence of reaction products which interfere with the SAE determination.

9.11 Effect of enzymatic treatment on canola meal proteins

The evaluation of the new enzymatic treatment would not be complete if the effect of the new process on the nutritional quality of the meal is not addressed. Considering that the most valuable components of the meal are proteins, their properties were used to test the effect of the new process on the meal quality.

In order for proteins to be adopted by the food industry they should maintain or enhance the quality of the food in which they are used. Therefore, in addition to satisfactory nutritional properties, they must have acceptable color, flavor and texture, and should posses specific functional properties [Thompson *et al.*, 1982].

For this part of the study, four protein isolates were prepared from differently treated canola meals, namely the untreated meal (isolate M1), the meal extracted with MeOH (isolate M2), the meal obtained after enzymatic treatments of 10.0% (w/v) meal suspension (isolate M3), and of the meal with low moisture content (isolate M4). The tests performed on each isolate included determinations of nitrogen solubility, whipping capacity, foam stability and colour evaluation. A commercial bovine albumin was tested as the control. The obtained results are shown in Table 9-12.

The nitrogen solubility tests showed that the highest crude protein content was from the isolate M2 prepared from the MeOH extracted meal. It was followed by isolate M4 from the meal treated at low moisture content. The lowest crude protein content was obtained for isolate M3. However, it was only 5.0% lower than the crude protein content of isolate M1 obtained from the untreated meal.

When the whipping capacities were compared, it was found that isolate M2 showed the highest whipping capacity. Its volume increased 3 times after 6 min of whipping. This represents 20.0% higher capacity than the one of the bovine solution. The lowest whipping capacity was observed for isolates M4 and M1.

With respect to foam stability, it was found that the foam from isolate M2 was the most stable, followed by the foams from isolates M1, M4 and M3. Although the foams obtained from

Table 9-12. Functional properties of protein isolates from non-treated and enzymatically treated canola meal

Functional property	Protein isolate[d]				
	Bovine	M1	M2	M3	M4
Crude protein content [%]	-	69.63	80.71	66.16 71.71[a]	71.56
Whipping capacity % vol. increase	225	125	300	150	100
Foam stability[b]					
0.0 min	100.0	100.0	100.0	100.0	100.0
20 min	33.3	80.0	88.0	53.3	70.0
40 min	15.5	68.0	76.6	26.6	50.0
60 min	11.1	52.0	66.6	23.3	45.0
120 min	2.2	16.0	50.0	10.0	20.0
Colour[c]					
R	255	238	240	191	220
G	255	151	170	113	125
B	234	84	85	17	64
R[a]			240	252	249
G[a]			165	225	210
B[a]			89	201	168

[a] - isolate washed with acetone
[b] - % of foam remaining per given time
[c] - described by a combination of the Red, Green and Blue using 256 colour palette and the SVGA monitor.
[d] - proteins isolate prepared from: untreated meal - M1; MeOH extracted meal - M2; enzymatically treated meal at: high moisture content 91.0% - M3; and low moisture content 73.0% - M4

the isolates from the enzymatically treated meals were the least stable, their stabilities were still higher than that of bovine albumin.

The colour of the protein isolates M3 and M4 was light brown-red. This colour was similar to the one noticed for the final products of the enzymatic transformation of SA. It suggested that the products of the enzymatic conversion of SAE became attached to the canola meal proteins. Washing of the isolate with acetone removed all of the colour, producing the isolate with fairly identical color to that of bovine albumin. This should be considered as improvement of proteins quality because isolate M2 (MeOH extracted meal) retained its relatively dark colour after the same treatment.

The results shown in Table 9-12 suggest that, in general, the enzymatic treatment did not have any negative effects on the properties of the protein isolates. Addition of a simple acetone-washing step to the procedure for the preparation of phenolic-free protein isolates from the enzymatically treated meals resulted in an isolate with a desirable colour. The washing step also increased the crude protein content of the isolate.

Part IV

Conclusions and Recommendations

Chapter 10

Conclusions

The following is a list of conclusions that were reached in this project concerning the novel enzymatic method for the upgrading of canola meal. The conclusions are presented in groups pertaining to the particular part of the thesis.

10.1 Enzyme production

- The white-rot fungus *Trametes versicolor* secretes an enzyme preparation capable of transforming a vast range of phenolic compounds including sinapic acid and sinapine, the major phenolics found in canola meal. The enzyme is produced late in the growth cycle when the fungus is cultivated in a submerged process, in batch reactors, using a semi-defined medium. The presence of Vogel's minerals is essential for the enzyme production, with the optimum amount being 0.5% (v/v). The production of the enzyme preparation did not require addition of an inducer. However such a produced enzyme preparation had a three times lower activity towards ferulic acid than the preparation produced using 2,5-xylidine as an inducer.

10.2 Model enzymatic system

- In the presence of oxygen, the enzyme preparation transformed 3,5-dimethoxy,4-hydroxy cinnamic acid (sinapic acid, SA) and its derivatives including, sinapine (SIN), sinapaldehyde (SALD) and sinapyl alcohol (SALC). The optima reaction temperature and pH for SA, SIN and

SALD are: 60°C and 3.7; 50°C, 4.2; 50°C, 3.2, respectively. Higher concentrations of SA and SIN inhibited the enzyme, whereas inhibition with SALD was not observed. The enzyme was very thermostable; the maximum thermal stability was noticed at the pH values between 4.5 and 6.5. The enzyme was inhibited by sodium azide while its activity was not affected by EDTA. Based on the results obtained, the enzyme was characterized as a polyphenol oxidase.

- The enzymatic oxidation of SA takes place in two distinct stages, Phase A and Phase B. In Phase A, SA is transformed to r-1H2c,6c-bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione, also called dehydrodisinapic acid dilactone (DAD). The mechanism of this reaction consisted of the coupling of two phenoxy radicals by the β-β mode, and a subsequent intramolecular nucleophilic attack. In Phase B, DAD is transformed by the action of polyphenol oxidase with the formation of several intermediate products, including 4-(4-(3,5-dimethoxy-4-oxo-2,5-cyclohexadienyliden)-1,4-dihydroxy-(*E*)-2-butenylidene)-2,6-dimethoxy-2,5-cyclohexadien-1-one. One of the final products of the overall transformation of SA, *i.e.*, Phases A and B, is 2,6-dimethoxy-*p*-benzoquinone, however, its yield was only 23.0%.

- The mechanism of SA transformation by the enzyme preparation containing the polyphenol oxidase can be described either by the Ordered Bi-Bi or Theorell-Chance Bi-Bi mechanisms, with oxygen being the first substrate and SA as the second. The overall conversion of SA can be described by the Theorell-Chance Bi-Bi mechanism with three alternate oxidizable substrate, SA, DAD, and, possibly, 2,6-dimethoxy-*p*-hydroquinone that is formed during the thermolysis of DAD. The affinity of the enzyme preparation towards SA was higher than for DAD or 2,6-dimethoxy-*p*-hydroquinone. The Michaelis-Menten constants for oxygen and SA were found to be 0.143 and 0.018 mM, respectively, indicating that although the enzyme can be saturated with SA, true oxygen saturation is impossible at oxygen concentrations attainable under normal conditions.

- The enzymatic transformation of SIN by the enzyme preparation containing the polyphenol oxidase from *Trametes versicolor* can also be described by the Theorell-Chance Bi-Bi mechanism. The optimum temperature for this transformation (determined using a mathematical model) was 60°C even though 50°C was determined experimentally. The reason for this discrepancy is due to

the fact that the temperature affects the oxygen solubility which in turn affects the initial reaction rate. The optimum pH for this reaction changed from 4.25 at the lower temperature to 4.4 at 60°C.

10.3 Canola meal system

- The treatment of the commercial canola meal with the enzyme preparation secreted by the white-rot fungus *Trametes versicolor* ATCC 42530 resulted in a meal with the phenolic content decreased by 98.0%. The concentration of the soluble sinapic acid esters, SAE, dropped from the initial concentration of 22.2 g/kg to 0.44 g/kg after 1 hour of processing. In some cases, a phenolic-free meal was obtained. The content of free sinapic acid dropped below the detection limit, whereas the concentration of the insoluble-bound sinapic acid esters was decreased by 56.0%. The tannin content in the treated meal was also reduced by 50.0%.

- The enzymatic treatment resulted in a negligible amount of phenolics in the treated canola meals prepared in the laboratory from the seeds of a few cultivars namely, Eldorado, Ceres, Profit, Falcon, Cyclone.

- When investigating the new enzymatic process it was found that: (1) changing the pH of buffer used as the continuous phase in the process had a negligible effect on the extent of SAE decrease due to the natural buffering capacity of CM; (2) the system was saturated with the enzyme when the enzyme concentration was 4 nkat per mL of the continuous phase; (3) the optimum process temperature was 50°C.

- The enzymatic process was extraction-limited at low concentrations of SAE. This effect was reduced when 85.0% of the meal's cell-wall material was solubilized by the treatment of the meal with wall solubilizing enzymes.

- The enzymatic process can be carried out with the meal concentration as high as 49% (w/v), even at temperatures up to 110°C, due to the protective action of the canola meal particles and canola meal proteins that caused an increase, on the average fourfold, in the thermal stability

of the enzyme. The distribution of the enzyme through the meal was the limiting factor for the process carried out at high meal concentration.

- The enzymatic treatment of the meal can be carried out at a low moisture levels of the system (meal moisture content between 30-75%) in the presence of hexane as a main component of the continuous phase. In such a system, the decrease in the SAE content was greater than when the high meal concentration slurry was treated without hexane. One of the reasons for this was a decrease in the meal-aqueous phase uptake observed in the presence of hexane.

- The enzymatic process for upgrading the meal can be described by a mathematical model describing the extraction of a solute (the SAE) from the solid matrix (the meal) into a solution of finite volume in which an enzymatic reaction takes place. To account for the diffusion-limited final stages of the process, CM has to be considered as a mixture of two types of particles, both having the same diameter but different effective diffusivities.

- When compared with other methods reported in the literature for the decrease in the phenolic content of rapeseed meals the new enzymatic method was the most effective, followed by the lime treatment. Combination of the enzymatic step with the other methods further decreased the phenolic content.

- The acetone-washed protein isolates from the enzymatically treated canola meal had better properties than isolates obtained from untreated meal.

- The phenolic content in the untreated meals can be measured using either spectrophotometric (direct and chemical) or HPLC analytical methods. However, the spectrophotometric methods overestimated SAE content in the enzymatically treated meal by 7.0% to 20.0%. The sensitivity limit for the direct and chemical spectrophotometric methods used for the determination of SAE content in enzymatically treated canola meals were 2.67 and 1.47 g of phenolics per kilogram of the meal, respectively.

Chapter 11

Recommendations

In this work, the novel enzymatic method for the decrease of the phenolic content in the commercial canola meal was investigated. Based on the results obtained, the following recommendations are made:

- Combine the enzymatic treatment of the meal with the production of the enzyme using:

  (1) solid state fermentation (SSF);

  (2) submerged fermentation.

It is of interest to compare the characteristics of the enzymes produced by these methods, especially since the presence of different complex phenolic compounds in the canola meal may result in the induction of enzymes with completely new characteristics and properties.

- The enzymatic method should be investigated in combination with a process for the preparation of high quality protein isolates. For instance, such a process could be based on the membrane separation technique, with the enzyme being immobilized on the membrane.

- Studies of the products of the enzymatic transformation of SA, which are not extractable with organic solvents, should be carried out because these compounds account for almost 80.0% of the products from this reaction.

- An experimental apparatus allowing the measurement of the time-changes in the concentration of oxygen, in both the liquid and gas phases, in the amount of phenolics extracted to the bulk liquid, and in the concentration of the product(s) of the enzymatic reaction in the bulk liquid should be designed and built in order to obtained more representative values of the parameters in the formulated model.

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Appendix A

Effect of temperature on oxygen solubility and its implication on enzyme activity

If the enzyme utilizes oxygen either as a second substrate or as an activator, the effect of temperature on the enzyme activity that is measured in the experiment is not only related to the change in the reaction rate constants, but it also includes an effect due to the change in oxygen concentration. This effect can have a profound impact on enzyme activity depending on the conditions at which the enzymatic assay is carried out. Figure A-1 shows two of the possible scenarios that can take place when the enzymatic tests are carried out in aqueous solutions in the presence of air at atmospheric pressure. The increase in the temperature of the system causes a decrease in the concentration of dissolved oxygen (Figure A-1A). Therefore, if the enzymatic test is carried out closed to enzyme-oxygen saturation conditions, the situation represented by the solid line Figure A-1B, then the change in oxygen concentration has a negligible effect on the enzyme activity. However, if the enzyme is not saturated with oxygen (dashed line Figure A-1B), then the change in oxygen concentration will cause a significant change in the enzyme activity and, therefore, the measured effect of temperature will represent the effect of the two opposite phenomena, the increase due to the higher temperature and the decrease due to the lower oxygen concentration.

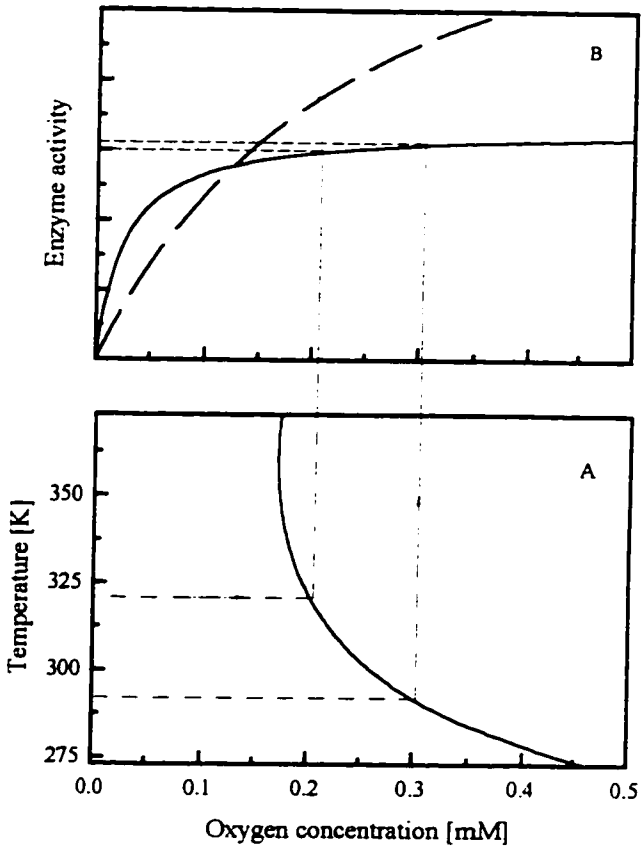

Figure A-1. Effect of the change in oxygen concentration caused by the change in system's temperature on the enzyme activity. (A) effect of temperature on the oxygen concentration; (B) - effect of oxygen concentration on enzyme activity assuming Michaelis-Menten type of enzyme kinetics. Solid and dashed lines represent higher and lower affinity of the enzyme for oxygen.

Appendix B

Derivations of the mathematical models

B.1 Deactivation of the native form of the enzyme

The deactivation of the stock solution of the enzyme can be described by the mechanism shown in Figure B-1, where E represents a native active form of the enzyme, E^p represents the postulated active, protected enzyme, and I stands for the inactivated enzyme. Transformations among these three forms of enzyme are described by the first order reactions.

$$E^p \underset{k_2}{\overset{k_3}{\rightleftharpoons}} E \xrightarrow{k_1} I$$

Figure B-1. Scheme for the deactivation of the enzyme stock solution.

The considered deactivation mechanism is described by the following system of ordinary differential equations:

$$\frac{dE}{dt} = -(k_1 + k_2)E + k_3E^p \tag{B.1}$$

$$\frac{dE^p}{dt} = -k_3E^p + k_2E \tag{B.2}$$

with the following initial conditions: $t = 0$, $E = C_0^T$ and $E^p = 0$, where C_0^T is the initial concentration of the active enzyme expressed in arbitrary units.

Solution of Eq.(B.1) and Eq.(B.2) with the above initial conditions gives the total concentration of the active enzyme, E_{tot}, after a given incubation time, t.

$$E_{tot} = E + E^p = C_1^T e^{-\lambda_1 t} + \left(C_0^T - C_1^T\right)e^{-\lambda_2 t} \tag{B.3}$$

where: $C_1{}^T$, λ_1, λ_2 are the model parameters that can be estimated from the experimental data regarding enzyme thermal stability. The respective rate constants can be calculated using the following formulas:

$$k_1 = \lambda_1 - \frac{C_1^T}{C_0^T}(\lambda_1 + \lambda_2) \;;\; k_3 = \frac{\lambda_1\lambda_2}{k_1} \;;\; k_2 = \lambda_1 + \lambda_2 - k_1 - k_3 \tag{B.4}$$

B.2 Theorell-Chance mechanism with three alternate substrates

The reaction scheme representing the enzymatic transformations of three substrates by the enzyme after its activation by a fourth substrate, each of these according to Theorell-Chance Bi-Bi mechanism, is shown in Figure B-2. The substrates for enzyme E are A, B, C and D. The products of the reaction are marked as P1, P2 and P3. Symbols EA, EQ, represent enzyme-substrate and enzyme-product complexes, respectively.

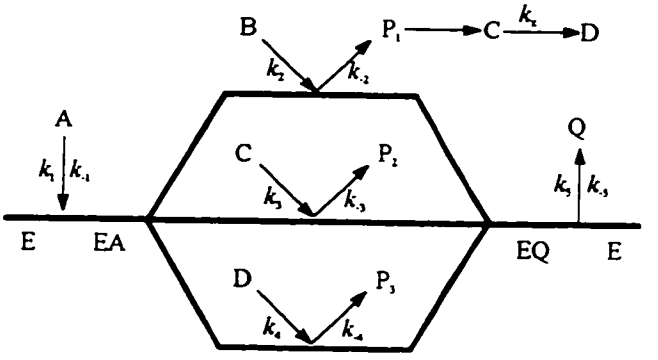

Figure B-2. Reaction scheme representing the Theorell-Chance Bi-Bi mechanism with three alternate substrates for the enzyme activated by the substrate A. Substrates C and D are the product of the transformation of substrate B.

The basic King-Altman figure representing the enzymatic part of the reaction scheme is shown in Figure B-3.

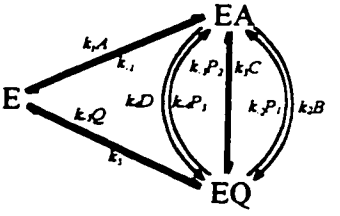

Figure B-3. Basic King-Altman figure for the Theorell-Chance Bi-Bi mechanism with three alternate substrates

The relative proportion of the total enzyme concentration, $[E]_t$, represented by a given species of enzyme, $[EX]$, is given by:

$$\frac{[EX]}{[E]_t} = \frac{\text{the sum of z terms}}{\text{a denominator}} \tag{B.5}$$

where: z is the number of acceptable (n-1) lined patterns on the King -Altman figure.

The numerator in Eq.(B.5) will contain the terms for the formation of EX by each King-Altman inter-conversion pattern (Figure B-3). The denominator will be the same for all enzyme species and equal to the sum of all numerator terms for all enzyme species. A detailed explanation of this procedure is given in Segel [1975].

Consequently, for the reaction presented in Figure B-3, the respective fractions of the total enzyme given by the particular enzyme species are

$$\frac{[E]}{[E]_t} = \frac{k_{-1}(k_5 + k_{-2}[P_1] + k_{-3}[P_2] + k_{-4}[P_3]) + k_5(k_2[B] + k_3[C] + k_4[D])}{\text{denominator}} \tag{B.6}$$

$$\frac{[EA]}{[E]_t} = \frac{k_1 k_5[A] + (k_{-2}[P_1] + k_{-3}[P_2] + k_{-4}[P_3])(1 + k_{-5}[Q])}{\text{denominator}} \tag{B.7}$$

$$\frac{[EQ]}{[E]_t} = \frac{k_{-1}k_{-5}[Q] + (k_2[B] + k_3[C] + k_4[D])(k_1[A] + k_{-5}[Q])}{\text{denominator}} \tag{B.8}$$

In the absence of products Eqs.(B.6)-(B.8) simplify to:

$$\frac{[E]}{[E]_t} = \frac{k_{-1}k_5 + k_5(k_2[B] + k_3[C] + k_4[D])}{\text{denominator}} \tag{B.9}$$

$$\frac{[EA]}{[E]_t} = \frac{k_1 k_5[A]}{\text{denominator}} \tag{B.10}$$

$$\frac{[EQ]}{[E]_t} = \frac{k_1[A](k_2[B] + k_3[C] + k_4[D])}{\text{denominator}} \tag{B.11}$$

The denominator is then equal to the sum of all the numerator terms. Thus, grouping similar terms:

$$\text{denominator} = k_{-1}k_5 + k_1 k_5[A] + (k_1[A] + k_5)(k_2[B] + k_3[C] + k_4[D]) \tag{B.12}$$

The differential rate equations for each substrate shown in Figure B-2 are given by:

$$-\frac{d[A]}{dt} = k_1[A][E] - k_{-1}[EA] \tag{B.13}$$

$$-\frac{d[B]}{dt} = k_2[B][EA] \tag{B.14}$$

$$-\frac{d[C]}{dt} = k_3[C][EA] \tag{B.15}$$

$$-\frac{d[D]}{dt} = k_4[D][EA] \tag{B.16}$$

On substitution of the respective formulas for the given enzyme species, Eqs.(B.13)-(B.16) become:

$$-\frac{d[A]}{dt}=\frac{k_1k_5[A](k_2[B]+k_3[C]+k_4[D])[E]_t}{k_{-1}k_5+k_1k_5[A]+(k_1[A]+k_5)(k_2[B]+k_3[C]+k_4[D])} \tag{B.17}$$

$$-\frac{d[B]}{dt}=\frac{k_1k_2k_5[A][B][E]_t}{k_{-1}k_5+k_1k_5[A]+(k_1[A]+k_5)(k_2[B]+k_3[C]+k_4[D])} \tag{B.18}$$

$$-\frac{d[C]}{dt}=\frac{k_1k_3k_5[A][C][E]_t}{k_{-1}k_5+k_1k_5[A]+(k_1[A]+k_5)(k_2[B]+k_3[C]+k_4[D])} \tag{B.19}$$

$$-\frac{d[D]}{dt}=\frac{k_1k_4k_5[A][D][E]_t}{k_{-1}k_5+k_1k_5[A]+(k_1[A]+k_5)(k_2[B]+k_3[C]+k_4[D])} \tag{B.20}$$

Dividing Eqs.(B.17)-(B.20) by the factor $k_1k_2k_5[A][B]$, and defining the following enzyme kinetic constants for Theorell-Chance mechanisms:

$$K_{i,A}=\frac{k_{-1}}{k_1};\ K_{m,A}=\frac{k_5}{k_1};\ K_{m,B}=\frac{k_5}{k_2};\ K_{m,C}=\frac{k_5}{k_3};\ K_{m,D}=\frac{k_5}{k_4};\ V_{max}=k_5[E]_t \tag{B.21}$$

and taking into consideration that the substrate C is the product from the enzymatic reaction of substrate B and, that the product D is formed by the first order conversion of C, the system of differential equation describing the reaction scheme shown in Figure B-2 is given by:

$$-\frac{d[A]}{dt}=\frac{V_{max}\left(1+\frac{K_{m,B}}{K_{m,C}}\frac{[C]}{[B]}+\frac{K_{m,B}}{K_{m,D}}\frac{[D]}{[B]}\right)}{\frac{K_{i,A}}{[A]}\frac{K_{m,B}}{[B]}+\frac{K_{m,B}}{[B]}+\left(1+\frac{K_{m,A}}{[A]}\right)\left(1+\frac{K_{m,B}}{K_{m,C}}\frac{[C]}{[B]}+\frac{K_{m,B}}{K_{m,D}}\frac{[D]}{[B]}\right)} \tag{B.22}$$

$$-\frac{d[B]}{dt}=\frac{V_{max}}{\frac{K_{i,A}}{[A]}\frac{K_{m,B}}{[B]}+\frac{K_{m,B}}{[B]}+\left(1+\frac{K_{m,A}}{[A]}\right)\left(1+\frac{K_{m,B}}{K_{m,C}}\frac{[C]}{[B]}+\frac{K_{m,B}}{K_{m,D}}\frac{[D]}{[B]}\right)} \tag{B.23}$$

$$-\frac{d[C]}{dt}=k_x[C]+\frac{V_{max}\left(\frac{K_{m,B}}{K_{m,C}}\frac{[C]}{[B]}-1\right)}{\frac{K_{i,A}}{[A]}\frac{K_{m,B}}{[B]}+\frac{K_{m,B}}{[B]}+\left(1+\frac{K_{m,A}}{[A]}\right)\left(1+\frac{K_{m,B}}{K_{m,C}}\frac{[C]}{[B]}+\frac{K_{m,B}}{K_{m,D}}\frac{[D]}{[B]}\right)} \tag{B.24}$$

$$-\frac{d[D]}{dt} = -k_x[C] + \frac{V_{max}\dfrac{K_{m,B}}{K_{m,D}}\dfrac{[D]}{[B]}}{\dfrac{K_{i,A}}{[A]}\dfrac{K_{m,B}}{[B]} + \dfrac{K_{m,B}}{[B]} + \left(1+\dfrac{K_{m,A}}{[A]}\right)\left(1+\dfrac{K_{m,B}}{K_{m,C}}\dfrac{[C]}{[B]} + \dfrac{K_{m,B}}{K_{m,D}}\dfrac{[D]}{[B]}\right)} \qquad \text{(B.25)}$$

The above equations, after rearrangement and introduction of the stoichiometry of the reactions, can be used to describe Phase A and Phase B of sinapic acid transformation by the polyphenol oxidase from *T. versicolor*.

B.3 Thermal stability of the enzyme in the presence of canola meal

The scheme representing the mechanism of deactivation of the enzyme in the presence of canola meal is presented in Figure B-4. According to the proposed mechanism, the enzyme, upon exposure to higher temperatures, changes irreversibly to the inactive form, I, *via* two independent pathways. One pathway involves a direct irreversible deactivation, while the other includes the formation of the intermediate active form (E_p) that will eventually also irreversibly deactivate to I, but at a different rate. Thus, at any time of the incubation process the total concentration of the active enzyme is a sum of the concentrations of the two active forms. All the steps in the proposed mechanism are first order reactions with the rate constants k_1, k_2 and k_3 for the direct deactivation (step 1), the formation of the intermediate form (step 2), and the deactivation of the intermediate form (step 3), respectively.

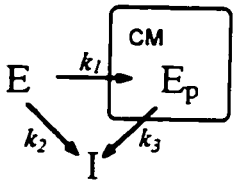

Figure B-4. Mechanism of enzyme deactivation in the presence of canola meal.

A differential equation describing the changes in the concentration of the native form of the enzyme with time is given by Eq.(B.26). Similarly, the equation representing the changes in the concentration of the intermediate form is given by Eq.(B.27).

$$\frac{dE}{dt} = -(k_1 + k_2)E \qquad \text{(B.26)}$$

$$\frac{dE_p}{dt} = -k_3E_p + k_1E \qquad \text{(B.27)}$$

Integration of Eq.(B.26) with the usual initial conditions: $t = 0$, $E = E_0$, yields:

$$E = E_0e^{-(k_1+k_2)t} \qquad \text{(B.28)}$$

Substituting Eq.(B.28) into Eq.(B.27), and solving the resulting equation using an integration factor $e^{\int k_3 dt}$ and the following initial condition: $t = 0$, $E_p = 0$; gives the concentration of the intermediate form of the active enzyme at any time (Eq.(B.29)).

$$E_p = \frac{k_1 E_0}{k_3 - k_1 - k_2}\left[e^{-(k_1+k_2)t} - e^{-k_3 t}\right] \tag{B.29}$$

Finally, combining Eq.(B.28) and Eq.(B.29) the expression for the overall concentration of the active enzyme, native plus intermediate, at any time of incubation is obtained.

$$E_{CM} = E + E_p = E_0\left[e^{-r_2 t} + \frac{r_1}{r_3 - r_2}\left(e^{r_2 t} - e^{r_3 t}\right)\right] \tag{B.30}$$

where: $r_1 = k_1$; $r_3 = k_3$; $r_2 = k_1 + k_2$.

B.4 Modeling of the enzymatic process to decrease the phenolic content in CM

B.4.1 Derivation of the reaction rate equations for canola meal system

The rates of the enzymatic reactions taking place in the canola meal system are derived assuming that: (1) the enzymatic transformation of the SAEs extracted from the canola meal is described by a single reaction proceeding according to the Theorell-Chance Bi-Bi mechanism; (2) the products from SAE transformations, P_{CM}, are also substrates for the enzyme, which compete with the remaining SAEs for the enzyme active site; (3) the transformation of all the products and other intermediates can be described by the single enzymatic step that proceeds according to the Theorell-Chance Bi-Bi mechanism.

Considering the above, the rates of the respective reactions can be obtained using the equations derived for the system representing the enzymatic transformation of SA (section B-2), after making the following substitutions: A = O_2; B = SAE; C = P_{CM}; $k_x = 0$; D = 0. These rate equations describing the CM system are given by:

$$r_{O_2} = \frac{\beta k_{5,CM} E_{CM} C'_{O_2}\left(K_{m,P_{CM}} C_{SAE} + K_{m,SAE} C_{P_{CM}}\right)}{(C'_{O_2} + K_{m,O_2,CM})(K_{m,P_{CM}} C_{SAE} + K_{m,SAE} C_{P_{CM}}) + K_{m,SAE} K_{m,P_{CM}} (C'_{O_2} + K_{i,O_2,CM})} \tag{B.31}$$

$$r_{SAE} = \frac{\beta k_{5,CM} K_{m,P_{CM}} E_{CM} C'_{O_2} C_{SAE}}{(C'_{O_2} + K_{m,O_2,CM})(K_{m,P_{CM}} C_{SAE} + K_{m,SAE} C_{P_{CM}}) + K_{m,SAE} K_{m,P_{CM}} (C'_{O_2} + K_{i,O_2,CM})} \tag{B.32}$$

$$r_{P_{CM}} = \frac{\beta k_{5,CM} E_{CM} C'_{O_2}\left(K_{m,SAE} C_{P_{CM}} - K_{m,P_{CM}} C_{SAE}\right)}{(C'_{O_2} + K_{m,O_2,CM})(K_{m,P_{CM}} C_{SAE} + K_{m,SAE} C_{P_{CM}}) + K_{m,SAE} K_{m,P_{CM}} (C'_{O_2} + K_{i,O_2,CM})} \tag{B.33}$$

where: $K_{m,i}$ - are the enzyme kinetic constant with respect to an i substrate: $C^l_{O_2}$ - concentration of oxygen in the liquid; E_{CM} concentration of the active enzyme given by Eq.(B.30); β- proportionality factor

B.4.2 Derivation of partition coefficient

The following is the derivation for the relationship between the apparent equilibrium concentration of the solute at the surface and its concentration in the bulk liquid.

From the equilibrium mass balance, the apparent concentration of the solute in the solid, w_t, that would be measured when the bulk of the solvent was removed, is given by Eq.(B.34):

$$w_t = \frac{V_p}{M_S} C_l + w^e_S \tag{B.34}$$

where: V_p is the total volume of the pores (cm^3), M_S is the mass of the solid (g), w_t is the apparent concentration of the solute in the solid (g_{solute}/g_{solid}), C_l is the concentration of the solute in the liquid (g_{solute}/cm^3), w^e_s is the equilibrium concentration of the solute in the solid (g_{solute}/g_{solid}).

Introducing the density of the solid and the apparent density of the liquid, Eq.(B.34) can be written

$$w_t = \varepsilon \frac{\rho_l}{\rho_s} w^e_l + f\left(w^e_l\right) \tag{B.35}$$

where: ρ_l and ρ_s represent densities of liquid and solid, respectively, w^e_l is the equilibrium mass concentration of the solute in the liquid ($g_{solute}/g_{solvent}$), ε is the porosity of the solid, and the function $f(w^e_l)$ represents sorption equilibrium and relates the equilibrium concentrations of the solute on the surface, w^e_s, and in the bulk liquid, w^e_l.

If the solid has immbibery properties, then the apparent concentration of the solute should be calculated using Eq.(B.36), derived in the same manner as Eq.(B.35).

$$w_t = \left(\frac{\varepsilon}{\rho_s} + MU\right) \rho_l w^e_l + f\left(w^e_l\right) \tag{B.36}$$

where: MU represent the solid-solvent uptake ($mL_{solvent}/g_{solid}$), defined as the volume of the solvent irreversibly bound/entrapped by a unit mass of the solid.

Introducing the partition coefficient, K_{iso}, defined as the proportionality factor between the apparent concentration of the solute in the solid, measured at equilibrium when the bulk solvent was removed, and the equilibrium concentration of the solute in the removed solvent, the apparent concentration of the solute in the solid is given by

$$w_t = K_{iso} w^e_l \tag{B.37}$$

Thus, after combining Eq.(B.36) with Eq.(B.37) the formula for the partition coefficient, K_{iso}, can be obtained. Depending on the type of isotherm selected to represent equilibrium of sorption $f(w^e_i)$, K_{iso}, can be either dependent or independent of the solute concentration in the bulk liquid. Concentration independent K_{iso} is obtained when a linear type of equilibrium isotherm is used, while a concentration dependent K_{iso} is obtained when the Langmuir or the Freundlich types of isotherm are used. Formulas for the partition coefficients when using linear, Langmuir and Freundlich type of sorption isotherm are presented in Table B-1 later in this Appendix.

B.4.3 Calculations of oxygen concentration

The changes in the concentration of the dissolved oxygen in the liquid phase observed during the enzymatic process depends on the rate of enzymatic reaction, and on the rate of the oxygen transfer from the gas phase to the bulk liquid. The latter process is governed by the oxygen transfer coefficient k_la and the oxygen equilibrium concentration at the gas-liquid interface, $C^*_{O_2}$. This concentration depends on the temperature, the composition of the liquid, and the partial pressure of oxygen in the gas phase. Neglecting the effect of salts on the oxygen solubility, the equilibrium concentration of dissolved oxygen at the interface, $C^*_{O_2}$, can be calculated using *Henry's law*.

$$C^*_{O_2} = \frac{\rho_l X^{gas}_{O_2} P}{k_H Mw_{H_2O} - X^{gas}_{O_2} P\left(Mw_{H_2O} - Mw_{O_2}\right)} \tag{B.38}$$

where: k_H is the Henry's constant

The changes in the concentration of oxygen (expressed in mole fractions) in the gas phase in a closed system occurring due to the utilization of oxygen in the liquid phase, are described (assuming the constant volume of the gas phase and neglecting pressure changes) by the following equation

$$\frac{dX^{gas}_{O_2}}{dt} = -\frac{RT}{P}\frac{V_l}{V_g} k_l a\left(C^*_{O_2} - C^l_{O_2}\right) \tag{B.39}$$

Henry's constant, k_H (atm), for oxygen and water can be calculated at a given temperature using the equation proposed by Benson *et al.* [1979], and recommended by Battino [1981]:

$$\ln k_H = 3.71814 + 5.59617 \times 10^3 \frac{1}{T} - 1.049668 \times 10^6 \frac{1}{T^2} \tag{B.40}$$

where: T is the temperature (K).

B.4.4 Model development

The dynamics of the enzymatic decrease of the phenolic content in canola meal is described, using the assumptions presented in Chapter 9: section 9.6, by the following system of coupled ordinary differential equations(ODE) and partial differential equations (PDE):

Solid phase

$$\frac{D_{eff,i}}{r_i^2}\frac{\partial}{\partial r}\left(r_i^2\frac{\partial w_i}{\partial r}\right)=\frac{\partial w_i}{\partial t} \quad ; \; 0\le r_i\le R_i \; ; \; t\ge 0 \tag{B.41}$$

$$3M_{S,i}\frac{D_{eff,i}}{R_i}\frac{\partial w_i}{\partial r}=N_{0,i} \quad ; \; r_i=R_i \; ; \; t>0 \tag{B.42}$$

with the following initial conditions:

$$w_i=f_i(r) \; ; \; 0\le r_i\le R_i \; ; \; t=0 \tag{B.43}$$

$$w_i=f\left(C_{SAE}\right) \; ; \; r_i=R_i \; ; \; t>0 \tag{B.44}$$

$$\frac{\partial w_i}{\partial r}=0 \; ; \; r_i=0 \; ; \; t>0 \tag{B.45}$$

Liquid phase

$$\frac{dC_{SAE}}{dt}=-\frac{1}{V_l}\sum_{i=1}^{I}\frac{N_{0,i}}{Mw_{SAE}}-r_{SAE} \; ; \; t>0 \tag{B.46}$$

with the following initial conditions:

$$C_{SAE}=0 \; ; \; t=0 \tag{B.47}$$

For the sake of clarity, the differential mass balances of oxygen in the gas and liquid phases, and the differential mass balance for the product of the enzymatic transformation of SAE were omitted. The resulting differential equations, although a part of the overall model (section 9.6), do not contribute to the method of solution presented in this section. In addition, only a single particle is considered.

B.4.5 Method of solution

The method of solution used in this work is based on the work of Papathanasiou *et al.* [1986] who modeled the dynamic response of an immobilized cell/enzyme CSTR. This method involves the following steps:

- Find a solution for Eq.(B.41) based on the existing analytical solution for the case of constant surface condition, applying *Duhamel's theorem*.
- Evaluate the mass flux of SAE on the surface of CM particle, $N_{0,i}$, that is, evaluate the partial derivative $(\partial w_i/\partial r)_{r=R}$.
- Substitute evaluated $N_{0,i}$ in Eq.(B.46) and solve the resulting ODE using a standard numerical method.

B.4.6 Solution of Eq.(B.41) based on the *Duhamel's theorem*

The solution of the differential equation representing the diffusion from a sphere for the time-dependent boundary condition can be obtained using the solution of the same equation for the constant boundary condition by applying *Duhamel's theorem* [Carslaw and Jaeger, 1959 p.233], namely:

"If $v=F(r,\varphi,\theta,t)$ represents the concentration at (r,φ,θ) at the time t in a solid in which the initial concentration is zero, while its surface is kept at concentration unity, then the solution of the problem when the surface concentration is given by $\phi(t)$, is given by [Eq.(B.48)]" [Carslaw and Jaeger, 1959]

$$\vartheta(r,\ \varphi,\ \theta,\ t) = \int_0^t \phi(\lambda)\frac{\partial}{\partial t}F(r,\ \varphi,\ \theta,\ t-\lambda)d\lambda \tag{B.48}$$

The solution of Eq.(B.41) for the constant surface condition, $w_i(R_i,t)=w1_i$, and for the uniform initial distribution of SAE in the meal, $w_i(r,0)=w0_i$, is given by [Carslaw and Jaeger, 1953 p.235; Crank, 1983 p.91]:

$$w_i^*(r,t) = w1_i + (w1_i - w0_i)\frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}e^{-D_{eff,i}\left(\frac{n\pi}{R_i}\right)^2 t}\sin\left(\frac{n\pi r}{R_i}\right) \tag{B.49a}$$

Eq.(B.49) can be rewritten as follows:

$$w_i^*(r,t) = -\frac{2R_i}{\pi r}w0_i\sum_{n=1}^{\infty}\frac{(-1)^n}{n}e^{-k_{n,i}t}\sin\left(\frac{n\pi r}{R_i}\right) + w1_i\left(1+\frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}e^{-k_{n,i}t}\sin\left(\frac{n\pi r}{R_i}\right)\right) \tag{B.49b}$$

where:

$$k_{n,i} = D_{eff,i}\frac{n^2\pi^2}{R_i^2} \tag{B.50}$$

The first part of Eq.(B.49b) represents the solution to the diffusion problem when the surface concentration is zero and the initial distribution of the solute is uniform and equal to $w0_i$. The second part, *2ndPart*, represents the solution to the same problem but with a zero initial concentration, and the surface concentration equal to $w1_i$. Therefore, if the surface concentration varies with time, *i.e.*, $w1_i=f(t)$, then the solution of the second part of Eq.(B.49a) can be obtained using the *Duhamel's theorem*. On doing so, the second part of Eq.(B.48a) becomes:

$$\begin{aligned} 2nPart &= \int_0^t \phi_i(\lambda)\frac{\partial}{\partial t}\left(1+\frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}e^{-k_{n,i}(t-\lambda)}\sin\left(\frac{n\pi r}{R_i}\right)\right)d\lambda = \\ &= -\frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\sin\left(\frac{n\pi r}{R_i}\right)k_{n,i}\int_0^t \phi_i(\lambda)e^{k_{n,i}(\lambda-t)}d\lambda \end{aligned} \tag{B.51}$$

After integrating by parts, Eq.(B.51) becomes:

$$2nPart = -\frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\sin\left(\frac{n\pi r}{R_i}\right)\left[\phi_i(t) - \phi_i(0)e^{-k_{n,i}t} - \Psi_{n,i}(t)\right] \tag{B.52}$$

where:

$$\Psi_{n,i}(t) = \int_0^t \frac{\partial\phi_i(\lambda)}{\partial\lambda}e^{-k_{n,i}(t-\lambda)}d\lambda \tag{B.53}$$

Consequently, after substituting Eq.(B.52) back into Eq.(B.49) and rearranging, the solution of Eq.(B.41), for the constant initial concentration, $w0_i$, and the time dependent surface concentration, $\phi(t)$, is given by:[*]

$$w_i(r,t) = \phi_i(t) - \frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\left[\left(w0_i - \phi_i(0)\right)e^{-k_{n,i}t} - \Psi_{n,i}(t)\right]\sin\frac{n\pi r}{R_i} \tag{B.54}$$

Considering that the concentration of the SAE in the meal is measured as the average value, it is convenient to express the concentration of the solute as the average concentration within the spherical particle according to:

$$\overline{w}_i(t) = \frac{3}{R_i^3}\int_0^{R_i} r^2 w_i(r,t)dr \tag{B.55}$$

Substituting Eq.(B.54) into Eq.(B.55) and differentiating, the average concentration of the solute in the single particle is given by:

$$\overline{w}_i(t) = \phi_i(t) + \frac{6}{\pi^2}\left[\left(w0_i - \phi_i(0)\right)\sum_1^{\infty}\frac{e^{-k_{n,i}t}}{n^2} - \sum_1^{\infty}\frac{\Psi_{n,i}(t)}{n^2}\right] \tag{B.56}$$

B.4.7 Evaluation of the flux term, $N_{0,i}$

The flux term, $N_{0,i}$, given by Eq.(B.42) can now be evaluated by taking the partial derivative $(\partial w_i/\partial r)_{r=R}$ of Eq.(B.54).

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[*] Eq.(B.54) can be also derived using the general expression for the intraparticle concentration profile in a sphere as a function of time, t, and distance from the bead center, r, for the initial solute distribution $f(r)$, and the time dependent surface concentration, $\phi(t)$, given by Carslaw and Jaeger [1959].

$$\vartheta(r,t) = \frac{2}{rR}\sum_1^{\infty}e^{-k_{n,i}t}\sin\left(\frac{n\pi r}{R_i}\right)\left[\int_0^R r' f(r')\sin\frac{n\pi r'}{R}dr' - n\pi(-1)^n\int_0^t e^{k_{n,i}\lambda}\phi(\lambda)d\lambda\right]$$

The above equation was originally derived using the *Duhamel's theorem*. To obtain Eq.(B.54), integration by parts is required.

$$N_{0,j} = 3M_{S,j}\frac{D_{eff,j}}{R_i}\left(\frac{\partial w_i}{\partial r}\right)_{r=R} = 6M_{S,j}\frac{D_{eff,j}}{R_i^2}\sum_{1}^{\infty}\left[\left(w0_i - \phi_i(0)\right)e^{-k_{n,j}t} - \Psi_{n,j}(t)\right] \tag{B.57}$$

Substitution of Eq.(B.57) into Eq.(B.42) yields the expression for the concentration profile of the solute in the bulk liquid as a function of time.

$$\frac{dC_{SAE}}{dt} = -\frac{6D_{eff,j}}{Mw_{SAE}R_i^2}\frac{M_{S,j}}{V_l}\sum_{n=1}^{\infty}\left[\left(w0_i - \phi_i(0)\right)e^{-k_{n,j}t} - \Psi_{n,j}(t)\right] - r_{SAE} \tag{B.58}$$

B.4.8 Evaluation of function $\Psi_n(t)$

The solution of Eq.(B.58) requires the knowledge of function $\Psi_n(t)$, which is defined by Eq.(B.53). This function cannot be easily obtained unless the form of $\phi(t)$ is known, or iterative numerical integration procedures are employed. On the other hand, it is possible to express the function $\Psi_n(t)$ in terms of an ODE that can be simultaneously solved with Eq.(B.58) to obtain the time-profile for the solute in the bulk liquid. Differentiation of Eq.(B.53) with respect to time yields:

$$\frac{d\Psi_{n,j}}{dt} = -k_{n,j}\Psi_{n,j}(t) + \frac{d\phi_i(t)}{dt} \tag{B.59}$$

with the initial condition:

$$\Psi_{n,j}(0) = 0 \tag{B.60}$$

Solution by numerical integration of Eq.(B.59) and Eq.(B.58) allows one to obtain the time profile of the solute in the liquid and consequently, using the partition coefficient K_{iso}, the intraparticle concentration profile of the solute, as a function of time (t) and distance from the sphere center (r) (Eq.(B.54)).

However, the numerical solution of Eq.(B.54) and Eq.(B.58) requires the calculations of the infinite summation of function Ψ_n, which means that the infinite set of ODE has to be solved, which is not reasonable. This difficulty can, however, be overcome if a good approximation of the infinite summation is used.

B.4.9 Evaluation of the infinite series $\sum_{1}^{\infty}\Psi_n(t)$, $\sum_{1}^{\infty}\frac{\Psi_n(t)}{n^2}$ and $\sum_{1}^{\infty}\frac{e^{-k_n t}}{n^2}$

Papathanasiou *et al.* [1986] showed that the dynamics of the functions $\Psi_{n,i}$ allow evaluation of these functions using a quasi-steady state approximation. Considering Eq.(B.59), it is seen that the dynamics of the functions Ψ_n depend of the value of the coefficient ($1/k_{n,i}$). For large values of $k_{n,i}$, the functions $\Psi_{n,i}(t)$ follow the forcing function $\left(\frac{1}{k_{n,j}}\frac{d\phi_i}{dt}\right)$ and, therefore, it is reasonable to assume that for $k_{n,i} > k_{n0,i}$ (*i.e.*, $n > n_0$) the $\Psi_{n,i}$'s can be evaluated using the quasi-steady state approximation given by:

$$\Psi_{n,j}(t) = \frac{1}{k_{n,j}} \frac{d\phi_i(t)}{dt} = \frac{1}{n^2} \frac{R_i^2}{D_{eff,j}\pi^2} \frac{d\phi_i(t)}{dt} \tag{B.61}$$

The value of n_o depends on the choice of the user-specified tolerance for the accuracy of the approximation that is the base for the series solution of Eq.(B.54). In this work the n_0 was set to 11. This is the same value as the one used by Papathanasiou *et al.* [1987].

After the above consideration, the infinite summation $\sum_{1}^{\infty} \frac{\Psi_{n,j}(t)}{n^2}$ can be estimated as follows:

$$\begin{aligned} \sum_{n=1}^{\infty} \frac{\Psi_{n,j}}{n^2} &= \sum_{n=1}^{n_0} \frac{\Psi_{n,j}}{n^2} + \frac{R_i^2}{\pi^2 D_{eff,j}} \frac{d\phi_i}{dt} \sum_{n_0}^{\infty} \frac{1}{n^4} = \\ &= \sum_{n=1}^{n_0} \frac{\Psi_{n,j}}{n^2} + \frac{R_i^2}{\pi^2 D_{eff,j}} \frac{d\phi_i}{dt} \left(\sum_{1}^{\infty} \frac{1}{n^4} - \sum_{1}^{n_0} \frac{1}{n^4} \right) = \\ &= \sum_{n=1}^{n_0} \frac{\Psi_{n,j}}{n^2} + \frac{\pi^2 R_i^2}{90 D_{eff,j}} \frac{d\phi_i}{dt} \left(1 - \frac{S2_{n_0}}{S2_{\infty}} \right) \end{aligned} \tag{B.62}$$

where: $S2_{n0}$ and $S2_{\infty} = (\pi^4/90)$ are the summation of terms n^{-4} from 1 to n_0, and from 1 to ∞, respectively.

The approximation of the infinite summation $\sum_{1}^{\infty} \Psi_{n,j}(t)$ is given by Papathanasiou *et al.* [1986]:

$$\sum_{n=1}^{\infty} \Psi_{n,j} = \sum_{n=1}^{n_0} \Psi_{n,j} + \frac{R_i^2}{6 D_{eff,j}} \frac{d\phi_i}{dt} \left(1 - \frac{S1_{n_0}}{S1_{\infty}} \right) \tag{B.63}$$

where: $S1_{n0}$ and $S1_{\infty}=(\pi^2/6)$ are the summation of terms n^{-2} from 1 to n_0, and from 1 to ∞, respectively.

The approximation of the infinite summation of $\sum_{1}^{\infty} \frac{e^{-k_{n,j}t}}{n^2}$ is based on the same principle as the one used for the two approximations discussed above. Considering that for high values of n and t the exponent part of the summation converges to 1, it seems reasonable, from the computational effort point of view, to calculate only the first n_f terms of the sum, and to approximate the remaining terms by a constant factor. On doing so, the infinite summation becomes:

$$\sum_{1}^{\infty} \frac{e^{-k_{n,j}t}}{n^2} = \sum_{1}^{n_f} \frac{e^{-D_{eff,j}\frac{n^2\pi^2}{R^2}t}}{n^2} + \frac{\pi^2}{6} \left(1 - \frac{S1_{n_f}}{S1_{\infty}} \right) \tag{B-64}$$

B.4.10 Summary of solution

By definition the function $\phi_i(t)$ is equal to the surface concentration $w_i(R_i, t)$, and it can be expressed in terms of the SAE concentration in the bulk liquid using the partition coefficient, $K_{iso,i}$, (Eq.(B.37)). On making the appropriate substitutions and applying the boundary conditions, the following set of equations describing the enzymatic process to decrease the phenolic content in CM is obtained:

$$\frac{d\Psi_{n,j}}{dt} = -D_{eff,j}\frac{n^2\pi^2}{R_i^2}\Psi_{n,j}(t) + B_{iso,j}\frac{dC_{SAE}(t)}{dt} \tag{B.65}$$

$$n = 1,2,3,\ldots,n_0$$

$$\frac{dC_{SAE}}{dt} = \frac{-\frac{6}{Mw_{SAE}}\frac{M_S}{V_l}\sum_{i=1}^{J}\frac{x_i D_{eff,j}}{R_i^2}\left[\sum_{n=1}^{\infty}\left(w0_i - \phi_i(0)\right)e^{-k_{n,j}t} - \sum_{n=1}^{n_0}\Psi_{n,j}(t)\right] - r_{SAE}}{1+\frac{M_S}{V_l}B_{iso,j}\left(1-\frac{S1_{n0}}{S1_\infty}\right)} \tag{B.66}$$

$$w_i(r,t) = K_{iso,j}C_{SAE}(t)Mw_{SAE} - \frac{2R_i}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\left[\left(w0_i - \phi_i(0)\right)e^{-k_{n,j}t} - \Psi_{n,j}(t)\right]\sin\frac{n\pi r}{R_i} \tag{B.67}$$

with the initial conditions:

$$C_{SAE} = 0 \;;\; \Psi_{n,i} = 0 \;;\; t = 0 \tag{B.68}$$

The parameter $B_{iso,i}$ in Eqs.(B.65)-(B.66) depends on the type of adsorption isotherm used to represent the equilibrium condition at the surface of the CM particle, and is equal to the derivative of Eq.(B.37) with respect to the concentration of the solute in the bulk liquid. The formulas for the partition coefficients, $K_{iso,i}$, and the respective parameters $B_{iso,i}$ for three types of adsorption isotherm are given in Table B-1.

Considering that the concentration of the SAE in the meal is measured as the average value, without taking the particle size distribution into account, it is convenient to express the concentration of the SAE in the meal as the average concentration:

$$\overline{w}_{tot}(t) = \sum_{i=1}^{J}x_i K_{iso,j}Mw_{SAE}C_{SAE}(t) - \frac{Mw_{SAE}}{15}\sum_{i=1}^{J}x_i\frac{B_{iso,j}R_i^2}{D_{eff,j}}\left(1-\frac{S2_{n0}}{S2_\infty}\right)\frac{dC_{SAE}}{dt} + \\ + \sum_{i=1}^{J}x_i w0_i\left(1-\frac{S1_{nf}}{S1_\infty}\right) + \frac{6}{\pi^2}\sum_{i=1}^{J}x_i\left[\sum_{n=1}^{\infty}\frac{1}{n^2}w0_i e^{-k_{n,j}t} - \sum_{n=1}^{n_0}\frac{1}{n^2}\Psi_{n,j}(t)\right] \tag{B.69}$$

The above set of equations can be easily modified to obtain solution for any extraction problem either with or without the chemical reaction, as long as the system is characterized by the high Biot numbers.

Table B-1. Equations representing partition coefficient K_{iso} and parameter B_{iso} for three types of adsorption isotherms

	Adsorption isotherms		
	Linear	Langmuir	Freundlich
K_{iso}	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + k_{ads}$	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + \frac{k_{Lg}}{K_{Lg}+C_{SAE}Mw_{SAE}}$	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + k_F Mw_{SAE}\left(C_{SAE}\right)^{\frac{1-n_F}{n_F}}$
B_{iso}	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + k_{ads}$	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + \frac{K_{Lg}k_{Lg}}{\left(K_{Lg}+C_{SAE}Mw_{SAE}\right)^2}$	$\left(\frac{\varepsilon}{\rho_s}+MU\right)\rho_l + \frac{k_F}{n_F}Mw_{SAE}\left(C_{SAE}\right)^{\frac{1-n_F}{n_F}}$

Appendix C

Comparison of three methods for the determination of sinapic acid esters content in enzymatically treated canola meals

The reported methods for determination of sinapic acid esters can be classified into two groups that are based on spectrophotometric and separation techniques. The choice of the method may depend on its suitability and/or practicality, because each method has some advantages and disadvantages. The spectrophotometric methods require a short time for the analysis of samples, but they are not-specific. On the other hand, the separation methods are very specific but a longer time is required to prepare and to analyze the samples. Considering that this work was focused on the elimination of all of the phenolics present in the meal, the disadvantage associated with the spectrophotometric methods was not a problem and, therefore, these methods were originally applied to evaluate the extent of enzymatic treatment. In the beginning, a direct spectrophotometric method was used. It gives the phenolics concentration in terms of all SAE plus free SA. Unfortunately, the results obtained indicated that this method suffered from interference related to the other light absorbing compounds, most likely created during the process. A similar phenomenon was observed when the ChS method was used, although to a lesser extent. The principle of the both spectrophotometric methods is shown in Figure C-1, where the results of the analysis of the methanol extracts from an untreated CM and a treated CM using the spectrophotometric methods are displayed. Figure C-1a shows the

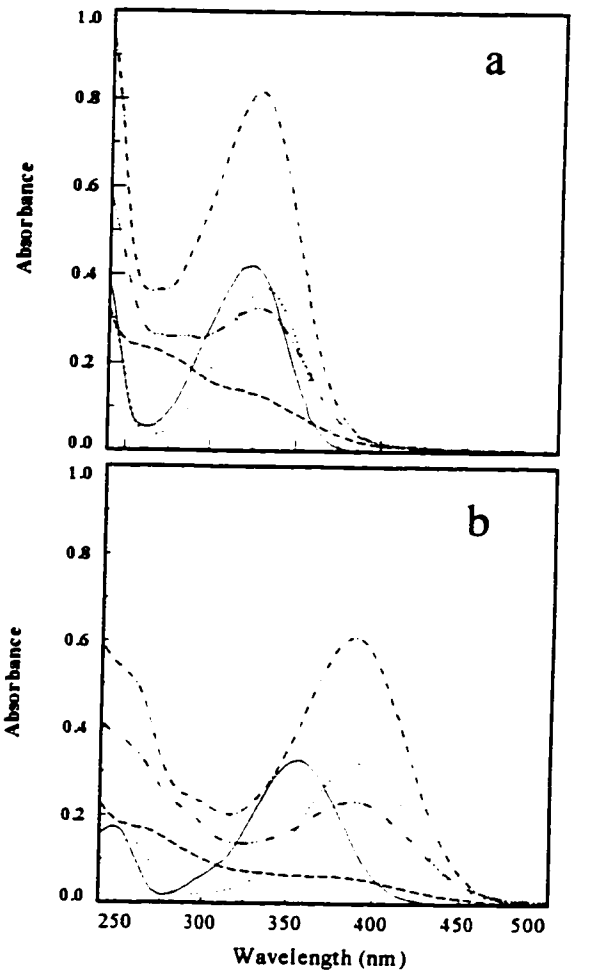


Figure C-1. Ultraviolet-visible spectra of the methanol extracts from: sinapine (·····), sinapic acid (———), untreated (-·-·-·-), and treated canola meal: buffer (-·····-·····-), enzyme (-------), before (a) and after (b) the addition of NaOH.

UV-visible spectra of the extracts as obtained by the DS method, whereas, Figure C-1b shows the spectra of the same extracts after the addition of NaOH (ChS method). To determine the specificity of each of the two methods, known solutions of SA and SIN (0.1mM) were also subjected to the analysis.

Direct spectrophotometric method. The principle of the direct spectrophotometric method is based on the changes in the magnitude of the absorbance at 330 nm with the changes in the phenolic concentration (Figure C-1a) and, therefore, does not account for the presence of other light absorbing substances. Consequently, the concentrations of SAE-SA measured by this method can be overestimated, especially in the case of the enzymatically treated meal when the products of enzymatic reaction have a significant absorbance at this wavelength. In such a case the curve representing a treated meal would in fact show the UV-visible spectra of the reaction products, and not necessarily of the residual SAE.

Chemical spectrophotometric method. The principle of the ChS method is presented in Figure C-1b. This method is based on the appearance of a bathochromic shift after the addition of NaOH to the solution of SAE and, therefore, is free from the interference observed with the DS method. ChS method is more sensitive than the DS method; it detected SIN in a concentration of 0.5 μg/mL, while the same concentration was hardly detectable by the DS method when a 1 cm spectrophotometric cuvette was used (Figure C-2). Although originally introduced only for the determination of total SAE concentration in the rapeseed meals (Mueller *et al.*, 1978a), the results obtained using this method also include the free SA [Łącki and Duvnjak, 1996b]. It was found that according to the ChS method 10 μg of SA would be detected as 1.75 μg of SIN (Figure C-1b). However, bearing in mind that the average concentration of the free SA in canola meals was usually around 10% of the SA liberated upon hydrolysis of all SAE [Krygier *et al.*, 1982], the overestimation of the SAE concentration was not bigger than 2.0% of all the SAE detected. Considering this, the ChS method was mainly used to evaluate the enzymatic process. However, regardless of the conditions of the process, meal pretreatment, and the time extent, the results obtained with the ChS method showed that the SAE content could not be decreased by more than 90.0%. This implied that either there were some SAEs that did not undergo enzymatic transformation, or there was an interference from unspecified sources which caused an overestimation of the SAE concentration in the treated meals.

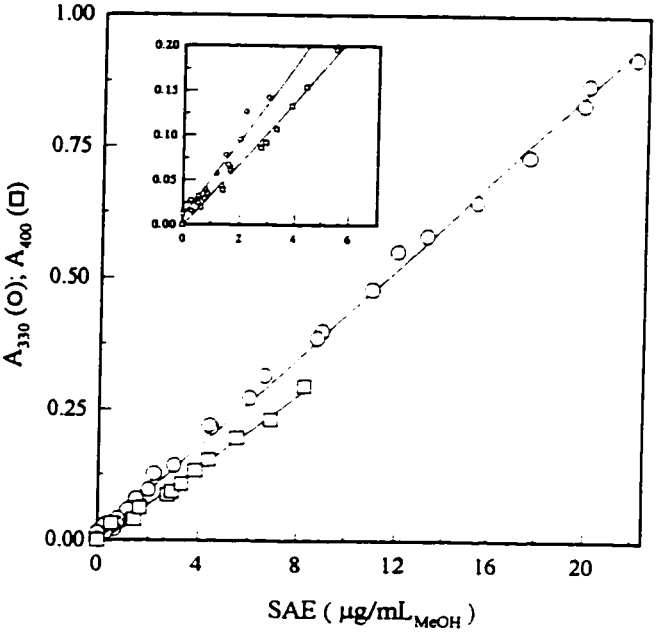


Figure C-2. Standard curves used for determination of sinapine using spectrophotometric methods: (O) - direct; (□) - chemical.

The existence of such an interference was observed during the determination of SAE content in the enzymatically treated meal. When performing these tests it was noticed that the absorbance of the methanol extract after the addition of NaOH was increasing, as opposed to an expected absorbance decrease associated with the unstability of a yellow complex formed (appearance of the peak at 400 nm shown in Figure C-3b). To show that the observed interference originated from the enzymatic process, the methanol extracts of the untreated and treated CM were diluted and concentrated, respectively to obtain two sets of SAE solutions with similar concentrations within each set. The absorbencies of these solutions after the addition of the base were recorded with time at 400 nm. The results are shown in Figure C-3 in terms of the difference between the absorbance at any given time and the initial absorbance instead of the actual absorbance values. The reason for this was that there was a large difference in the magnitude of the respective absorbencies for the measured concentrations. Curves (a) and (b) (Figure C-3) represent the results obtained with extracts from the untreated meal, either original (as extracted) or diluted, respectively. Both curves showed that regardless of the magnitude of the SEA concentration in the sample, the expected decrease in the absorbance was observed. The subsequent increase in the absorbance of the diluted sample (curve (b)) can be explained by the fact that an increase with a similar rate was observed for the blank solution (curve (c)). These two curves became almost parallel 7 seconds after the addition of the base. The discrepancy between this value and the one that can be read off the graph comes from the practical limitations that are associated with this method for it takes at least 2 seconds for the absorbance to be readable after the addition of NaOH. According to the pattern shown by curve (b), it would be expected that when the concentration of SAE in the treated CM was in the range of that represented by the diluted sample from the untreated meal (curve (b)), a similar curve should be obtained. However, when the extract from the treated meal was analyzed, instead of the decrease in the absorbance an increase was observed, curve (e), even though the sample turn yellow upon the addition of the base indicating the presence of SAE (Figure C-1b). This indicated that some sort of the interference was in fact present. The presence of this interference was further confirmed when the concentrated extract from the treated meal was analyzed (curve d). In this case the intensity of the yellow colour was identical to the one obtained with the extract from untreated meal, curve (a),

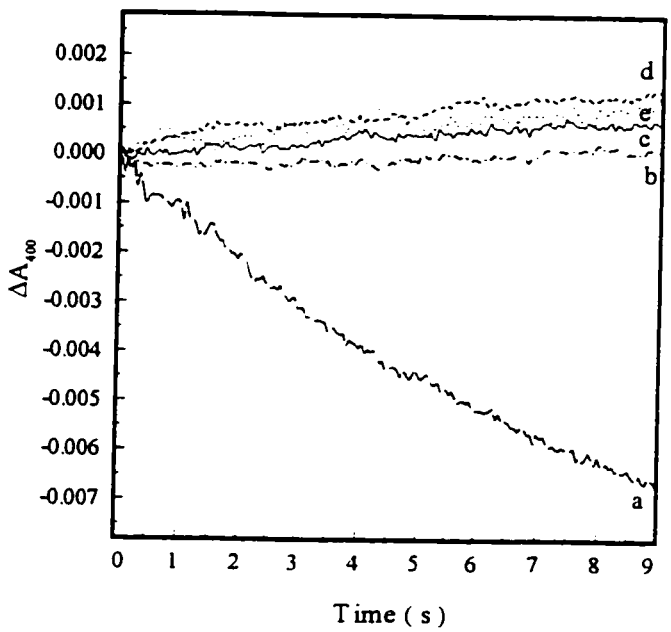


Figure C-3. Effect of time on the changes in the sample absorbance at 400 nm after the addition of NaOH: (a) untreated CM; (b) diluted untreated; (c) blank; (d) concentrated treated; (e) treated.

but still an increase in the absorbance was observed. The almost identical shapes of curves (e) and (d) could indicate that the former curve should be considered as a resultant of two opposite phenomena: a decrease due to the SAE present in the sample and an increase due to the interference from the side reactions of NaOH. These findings proved that some sort of interference was present and was related to the enzymatic treatment rather than the analytical method itself.

To eliminate the effects of the observed interference on the enzymatic process evaluation, a method based on a separation techniques had to be applied to measure the concentration of the SAE in the enzymatically treated meal.

Among the separation techniques, the method based on a reverse phase HPLC was considered the most suitable for the purpose of this work because of its simplicity and accuracy. The use of an ion-pair reagent, 1-heptanesulphonic acid, in the mobile phase [Henning, 1982; Clausen *et al.*, 1983; Bouchereau *et al.*, 1991] resulted in the elimination of the tailing phenomena encountered in the separation of amines using HPLC. This allowed the use of the same HPLC separation method to simultaneously quantitate the free phenolic acids and the choline esters.

HPLC method. The methanol extracts of the untreated CM meal were analyzed by the HPLC separation method (based on the method of Clausenn *et al.* [1983]). The chromatogram obtained is shown in Figure C-4. The separation data and UV spectra of the detected compounds are presented in Table E-2. The quality of the separation (Figure C-4) of SIN, peak C15, and SA, peak C11, obtained with this method is much better than the separation reported by Henning [1982]. In addition to these two compounds, 29 more peaks with relative concentrations not smaller than 1% of that of SIN, expressed in terms of absorbance at 328 nm, were detected. The fact that all of the considered compounds underwent enzymatic transformation (decrease in the area of respective peaks) (Figure 9-1) could indicate that all of the detected compounds, except SA, may be used for the calculation of SAE content, despite the fact that some of them may not be the true esters of sinapic acid. Because of the lack of the actual standards, except for SIN and SA, the possible character (identification) of all of the detected compounds was evaluated based on their UV spectra obtained using a PDA detector. As shown in Table E-2 most of the compounds have the maximum absorbance between 310 nm and 340 nm. This range of wavelength is characteristic for the cinnamic acid derivatives [Macheix *et al.*, 1990]. Furthermore, except for the compounds C1, C8, C12, C16-C20, C23, C28, and C31, all the other compounds showed a secondary absorbtion maximum at around 230-240 nm. Both of these wavelength ranges are characteristic for cinnamic acid choline esters [Bouchereau *et al.*, 1991]. Based on this information, the total SAE concentration was evaluated from the area of the peaks representing compounds C2-C7, C9-C11, C15, C16, C21, C22, C24-C27, C29, C30. Compounds C12, C17-C20, C31 were not considered since their UV spectra showed a particular band (shoulder) at 270-290 nm, that was followed by the maximum at around 320-330 nm. Such a shape could be associated with the derivatives of ferulic acid. Bouchereau *et al.* [1991] reported a difficulty in the separation of isoferuloylcholine and sinapine using a similar HPLC separation method. A very small difference in the retention time between SIN and the compound C17, in addition to the specific UV spectra of the latter one, could indicate that the compound C17 is isoferuloylcholine (FIN). None of these compounds could be a source of the interference detected using spectrophotometric method since, as shown in Figure 9-1c, all of them disappeared from the meal during the enzymatic treatment. This was expected since the free ferulic acid was transformed by this enzyme [Khan and Overend, 1990].

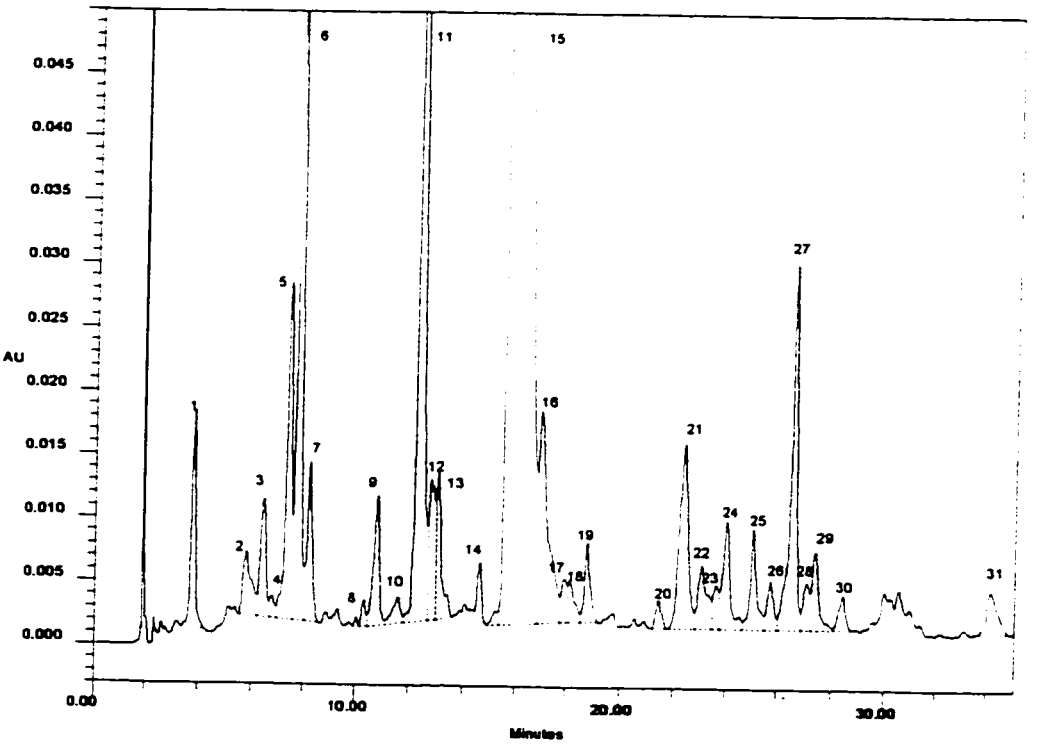


Figure C-4. Chromatogram of the methanol extract from the untreated canola meal. Peak information given in Table E-2. Detection wavelength: 328 nm. (the prefix 'C' used in the text for a peak identification was omitted in the figure for clarity).

Summarizing, the HPLC method used gave the most comprehensive results among the tested methods showing that as many as 31 compounds could contribute to the concentration of phenolic fraction in CM. This result is consistent with data reported by Bouchereau *et al.* [1991] who showed the existence of 29 cinnamic and benzoic acids choline esters in the methanol extracts from various rapeseeds. Any of the considered methods can be used for the measurement of the SAE content in untreated rapeseed meals, although, for the screening purposes, as more facile approach, both of the spectrophotometric methods should be recommended. On the other hand, the discrepancy between the results obtained by the three methods were observed when the enzyme-treated meals were analyzed. The products of enzymatic reaction could be responsible for an overestimation of the SAE concentration measured by the spectrophotometric methods. The sensitivity limits for the spectrophotometric methods are 2.67 and 1.446 grams of SAE per kilogram of the enzyme-treated meal for the direct and the chemical method, respectively.

Recalculations of the results obtained with ChS method.. Considering that the observed interference was proportional to the amount of SAE transformed by the polyphenol oxidase, and that the sensitivity of ChS was 1.446 g/kg, the following formula was used to recalculate the results obtained using ChS method.

$$C_{SAE}^{rec} = \frac{C_{SAE}^{buffer}}{C_{SAE}^{buffer} - C_{SAE}^{sl}} \left(C_{ChS} - C_{SAE}^{sl} \right) \qquad \text{(C-1)}$$

where: C_{SAE}^{corr} is the concentration of SAE in enzymatically treated meal, free from the interference caused by the product of the enzymatic reaction; C_{SAE}^{buffer} represents the concentration of SAE in the meal obtained at the same process condition using deactivated enzyme; C_{SAE}^{sl} is the sensitivity limit for the chemical spectrophotometric method; C_{ChS} is the concentration of SAE in the enzymatically treated meal measured with the chemical spectrophotometric method.

Appendix D

Method for analysis of reaction products using separation data obtained with PDA detector

Data obtained using an HPLC based separation method with Photo Diode Array (PDA) detector was analyzed with the Millennium Chromatography System manager version 2.1 for the presence of new peaks representing the products of the enzymatic reaction. The newest version of the Millennium software make it possible to subtract any two specified 3D chromatograms from each other. This program feature was introduced to eliminate effects of a mobile phase containing strong UV absorbing compounds when the separation is performed using a gradient elution method. It also allows the elimination of the presence of the system peaks or contaminants from the mobile phase. In the proposed procedure this ability of the software is employed differently. Instead of subtracting the blank run from all the samples, a 3D chromatogram data representing initial system conditions were subtracted. This action generated a transformed 3D chromatogram with positive and negative peaks representing the products and the substrates of reaction, respectively. The larger the peaks the greater is the extent of reaction. The obtained data are also free of any artifacts related to the character of the mobile phase. They also give robust means to distinguish reaction products: a 2D chromatogram (channel) extracted from the transformed 3D data will show products of the reaction as positive peaks (above the baseline). It should be noted that although this method is easy and straight forward with respect to a detection of products, it may lead to wrong conclusions if the retention times of the same substrate differ among the separation runs. In such a case, the appearance of the artifacts as positive peaks can be observed. Their presence can be relatively easily established since the peaks responsible for these artifacts will be accompanied by negative peaks, and the tips of these two peaks will be connected by a straight line (Figure D-1). However, a similar situation can be observed if a real product is formed that has the same, or almost the same retention time. Similarly, these kinds of peaks will be observed if an inert compound, and/or a less reactive isomeric form of the substrate is present in the system. The simplest way to detect the presence of this kind of artifact is the analysis of the respective UV-VIS spectra. If the spectra are the same with the isomerization being ruled out, then the positive peak will probably be an artifact. Another solution would be the addition of two internal standards to the sample being analyzed which would elute at the beginning and at the end of chromatogram. If the positive peaks are seen where the standards should be present, then the two considered separations differ in terms of retention times and the presence of the other artifacts is possible. On the other hand, if the standards cancel out then all of the positive peaks represent compounds that are either formed or do not undergo any transformation but have a similar retention time as the substrate.

The other very important aspect when applying this kind of analysis to the transformed 3D chromatogram is the choice of the wavelength at which the two-dimensional chromatogram will be extracted. Depending on the characteristics of the products, *i.e.*, their optical properties, a wrong choice of that wavelength may result in omitting all the information that the 3D data are providing. This phenomenon is shown using the data representing untreated and enzyme treated CMs. The transformed 3D chromatogram used in this example was obtained by subtracting results regarding untreated meal from these obtained after a 1 hour-long treatment. To show how the choice in the wavelength affects the analysis, five channels were extracted at 200 nm, 240 nm, 280 nm, 328 nm and 360nm (Figure D-2). The results showed that the higher was the wavelength, the fewer compounds were detected. Whereas, the chromatograms extracted at 200 nm and 240 nm showed 6 and 5 new peaks, D2-D7 and D2-D6, respectively, the rest of the derived channels showed only two peaks each. In addition, these pairs of peaks were not the same as at 280 nm (peaks D1 and D4) and the other two wavelengths (peaks D2 and D3). This finding showed that the products were absorbing light differently, and that the lower wavelengths should be used if only one-wavelength chromatogram is to be used. However, the Millennium software has some additional extended tools for extraction of the 2D channels. One of them is called Total Wavelength, and is based on averaging the absorbencies of all the wavelengths in the specified range. Applying this method the chromatogram shown in Figure D-4 was obtained. It shows that all six products were detected. Unfortunately, if the concentration of new compounds is low then the sensitivity of this method decreases owing to the averaging effect. It could result in a lack of detection of some of the products.

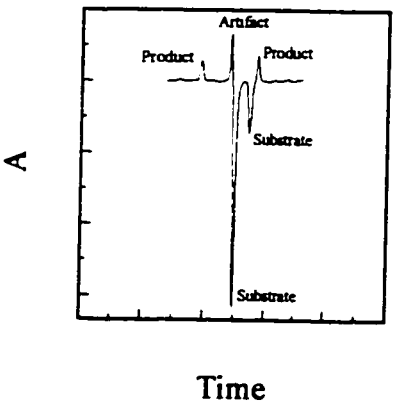


Figure D-1. Example of the artifact formed because of a difference in the retention time.

The other tool included in the Millennium software is based on the extraction of the 2D chromatogram called MaxPlot. This method creates the 2D channel where each data point is the maximum absorbance of the spectrum acquired at that point in time. This method is primarily used to maximize the sensitivity for integration and quantitation. However, when this method was applied to the derived 3D data set based on the subtraction of the initial sample (not the blank run!), it was found that it produced the best results from the product detection point of view (Figure D-3). This method is also sensitive to the starting wavelength and may fail to detect all of the products if that value is too high. As shown in Figure D-3 when the starting wavelength was 240 nm two fewer products were detected as compared to the case when MaxPlot was derived starting from 200 nm. One of the pitfalls of this method, however, is the lack of detection of the artifacts that are caused by the difference in the retention times in the sample that were used to prepare 3D data. Therefore, it should be recommended that spectra of the detected peaks should be closely examined in order to eliminate the possibility of misinterpretation of the results obtained.

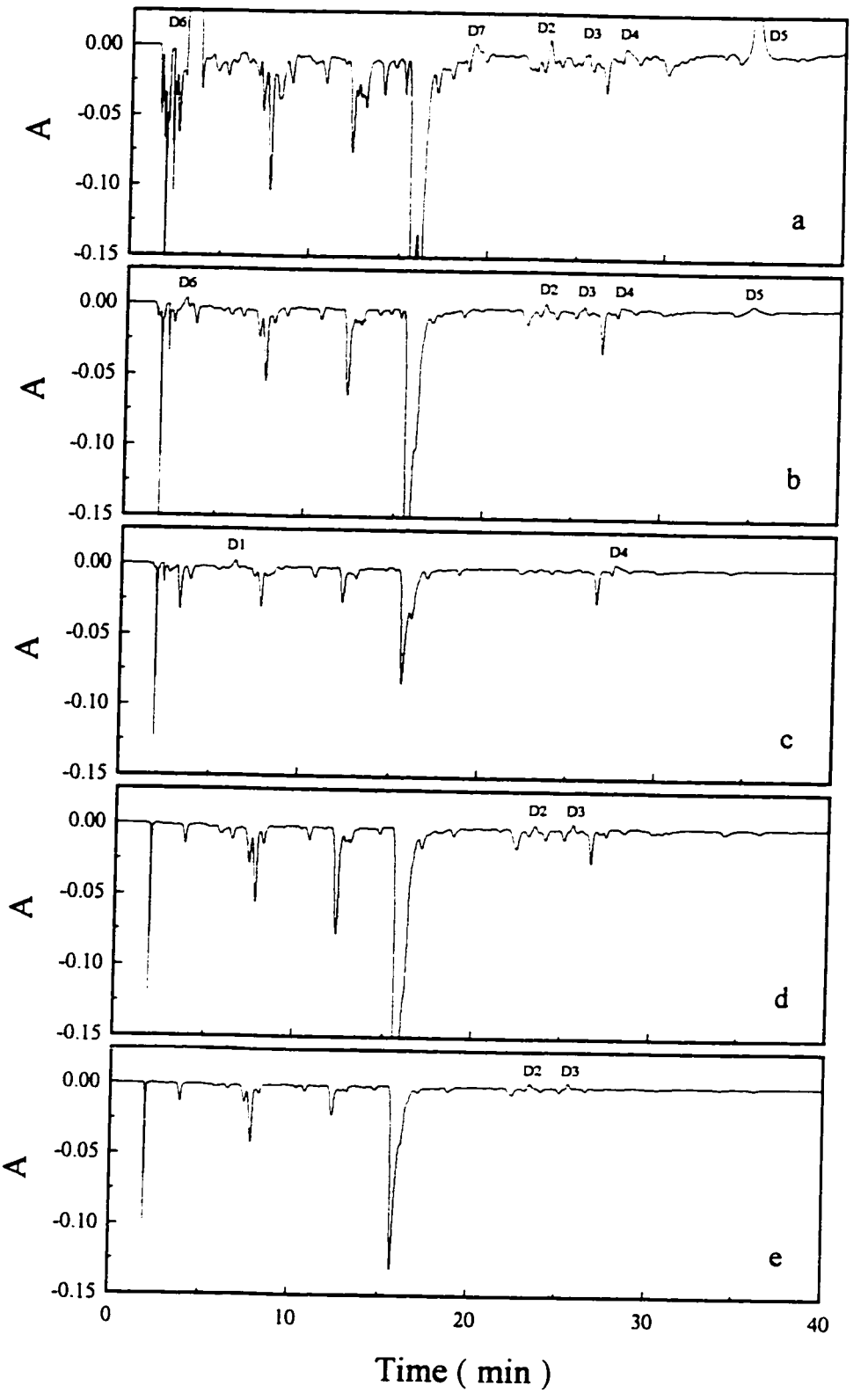


Figure D-2. Derived 2D chromatograms at constant wavelength from the transformed 3D data set obtained subtracting the sample representing untreated CM from the one representing enzymatically treated CM: (a) - 200 nm; (b) - 240 nm; (c) - 280 nm; (d) - 328 nm; (e) - 360 nm.

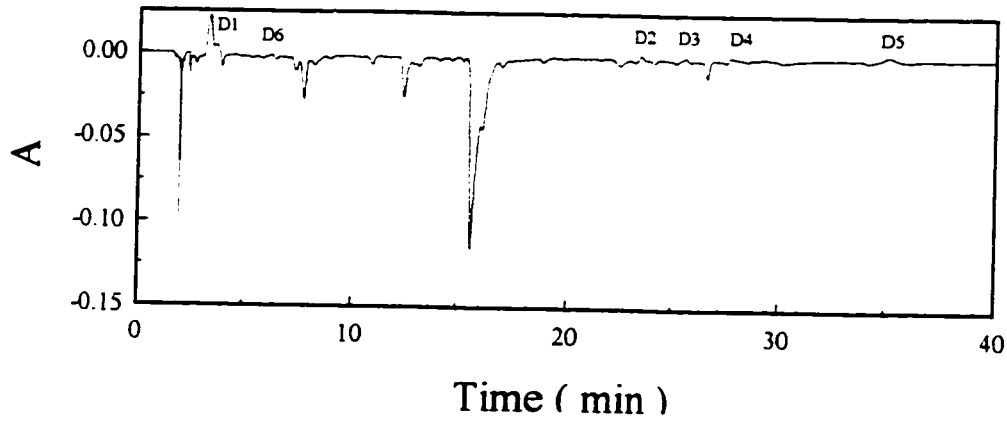


Figure D-4. Derived 2D chromatogram, the Total Wavelength, as extracted from 200 nm to 540 nm using the transformed 3D data set.

In summary, the use of HPLC data acquired using PDA detector can be analyzed in a robust way by subtracting the data representing the initial state of the system from these representing the analyzed sample and then extracting 2D MaxPlot from the obtained transformed-3D data set. The starting wavelength for the channel extraction should be set as low as possible. Care, however, should be taken to address the possible formation of the artifacts that are caused by the difference in the retention time

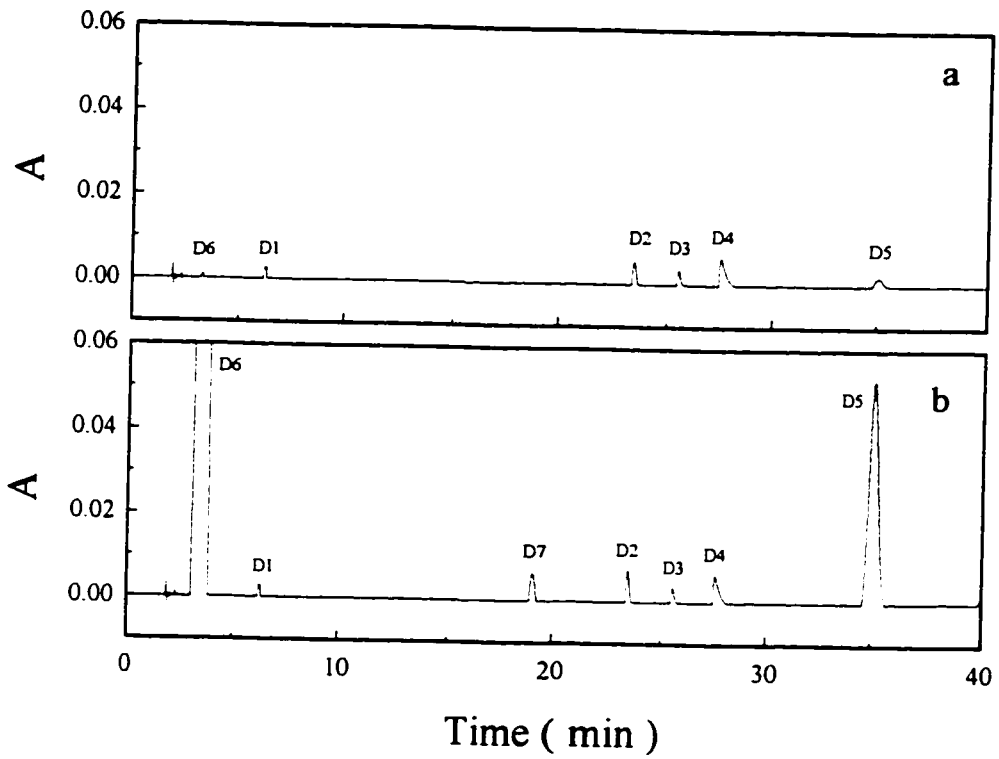


Figure D-3. Derived 2D chromatograms, MaxPlots, as extracted from: (a) - 200 nm to 540 nm; (b) - 240 nm to 540 nm using the transformed 3D data set.

between analyzing chromatograms. To check for the presence of these artifacts, an additional chromatogram should be extracted at the wavelength where the substrates absorb light. In addition, it can be suggested that the spectra for the new compounds should be taken using the original 3D data instead the one obtained from subtraction.

Appendix E

Tables of UV spectrophotometry, FTIR spectroscopy, Mass Spectrometry, NMR and X-ray Diffractometry data for phenolics from canola meal and the products from their transformation by an oxidase from T. versicolor

The following are the data obtained during the detection and characterization of the phenolic compounds present in canola meal, as well as the products of their transformations by the polyphenol oxidase from *T. versicolor*. Data are grouped into series representing specific analytical techniques used.

Table E-1 to Table E-3 include chromatographic and UV data for the model and CM systems, respectively.

Data obtained during MS analysis of the fractions collected during HPLC separation of the products from the enzymatic transformation of SA are presented in Table E-4. Graphical representation of the mass spectra for DAD and *p*-benzoquinone are presented in Figure E-2 and Figure E-1, respectively.

The IR spectra of the fractions collected during HPLC separation of the products from the enzymatic transformation of SA are presented in Figure E-3.

Proton ^{1}H and carbon ^{13}C NMR data for r-1H-2c,6c-bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione are given in Table E-5. The results are extracted from the NMR spectra of the sample representing the products of the enzymatic transformation of SA obtained after a 30 min long reaction. The HPLC chromatogram representing the composition of the sample as detected at 280 nm is shown in Figure E-4. The proton and carbon NMR spectra are shown in Figure E-5. The shifts presented in Figure E-5 were assigned to the respective carbons based on the comparison of the actual NMR spectra and the spectra generated by computer for DAD (Figure E-6).

The experimental details and the results from X-Ray crystallography analysis are shown in Table E-6 to Table E-8. These results are accompanied by the ORTEP drawings (Figure E-7) representing atom numbering in the structure of r-1H-2c,6c-Bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione.

E.1 Chromatographic data and UV-Vis spectra

Table E-1. Retention (capacity) factor and selectivity for the HPLC separation together with UV data for the compounds formed during the enzymatic transformation of SA

Peak	k'**	α**	λ (nm) and corresponding absorbance values***				
XI	8.445	2.004	208 (0.031)		249^{v} (0.002)	290 (0.020)	395 (0.001)
VI	9.858	1.728	208 (0.141)	235^{sh} (0.043)	260^{v} (0.006)	315 (0.036)	
III	11.299	1.029	208 (0.285)	241^{sh} (0.051)	260^{v} (0.010)	276^{sh} (0.007)	
IV	11.633	1.305	205 (0.125)	224^{p} (0.014)	241(0.014)	277^{v} (0.003)	346 (0.018)
X	11.895	0.710	203 (0.376)	229^{v} (0.011)	266 (0.012)		
I	12.624	1.168	208 (2.000)	234^{v} (1.141)	243 (1.191)	265^{p} (0.282)	276^{sh} (0.215)
XIII	14.308	1.082					
II	14.749	0.766	204 (2.000)	234^{v} (1.594)	243 (1.652)	265^{p} (0.433)	276^{sh} (0.298)
V	15.192	0.649	208 (0.146)	231^{v} (0.036)	242 (0.040)	287^{v} (0.006)	341 (0.027)
XIV	15.489		209 (0.022)	234^{v} (0.012)	247 (0.013)	270^{sh} (0.009)	288^{v} (0.004) 350 (0.015)
VIII	16.551	1.008	207 (0.130)	234 (0.059)	292^{sh} (0.022)		388^{v} (0.001) 420 (0.011) 339^{sh} (0.052) 353 (0.070)
IX	16.937	0.712	208 (0.512)	243^{sh} (0.112)	273^{sh} (0.033)	380^{v} (0.022) 295^{v} (0.017)	495^{sh} (0.182) 533 (0.230) 341 (0.038) 375^{v} (0.019)
XII	16.933	0.844	208 (0.987)	234^{v} (0.194)	243 (0.211)	428^{sh} (0.039) 277^{sh} (0.052)	492^{sh} (0.073) 533 (0.094) 334 (0.014) 425 (0.073)
VII	17.040	0.971	209 (0.094)			292^{sh} (0015) 501 (0.056)	525 (0.029) 392^{v} (0.011) 465^{sh} (0.035) 522^{v} (0.049) 569 (0.067)

* retention factor: $k' = (t_i - t_0) / t_0$; t_0 = 1.548 min.

** selectivity: $\alpha = k'_{i+1} / k'_i$, where i - peak number.

*** The absorbance values (in brackets) are obtained using PDA detector and are baseline corrected.

sh shoulder in the UV spectra rather then a maximum.

v valley in the UV spectra

p plateau in the UV spectra

Table E-2. Retention (capacity) factor and selectivity for the HPLC separation together with UV data for 31 compounds detected in the methanol extract of canola meal.

Peak #	k' *	α **	λ_{max} (nm) and corresponding absorbance values ***					
C1	1.002	2.034			265 (0.018)		325 (0.016)^{sh}	346 (0.02)
C2	2.038	1.162		238 (0.005)			323 (0.006)	
C3	2.370	1.077		238 (0.006)			328 (0.010)	
C4	2.553	1.126		238 (0.001)			328 (0.002)	
C5	2.875	1.069	200 (0.036)	238 (0.021)			333 (0.027)	
C6	3.075	1.076	200 (0.105)	228 (0.052)	266 (0.043)		333 (0.055)	
C7	3.311	1.328	200 (0.036)	224 (0.014)			328 (0.013)	
C8	4.399	1.059	200 (0.0015)	242 (0.001)				347 (0.002)
C9	4.661	1.089	200 (0.019)	219 (0.015)	266 (0.009)		318 (0.011)	
C10	5.079	1.072		242 (0.005)			318 (0.002)	
C11	5.445	1.043	200 (0.075)	238 (0.067)			323 (0.077)	
C12	5.680	1.010	200 (0.034)	233 (0.015)		290 (0.008)^{sh}	323 (0.011)	
C13	5.820	1.145	200 (0.043)	233 (0.016)			323 (0.012)	
C14	6.664	1.045	200 (0.006)	238 (0.0035)	266 (0.003)		333 (0.004)	
C15	7.187	1.099	200 (0.316)	238 (0.318)			328 (0.396)	
C16	7.902	1.060	200 (0.031)	228 (0.013)	270 (0.011)^{sh}		328 (0.017)	
C17	8.381	1.014	200 (0.014)	228 (0.006)	270 (0.003)^{sh}		328 (0.004)	
C18	8.503	1.038	200 (0.004)	233 (0.002)	271 (0.001)		328 (0.003)	
C19	8.834	1.103	200 (0.017)	228 (0.007)	271 (0.005)	290 (0.005)	323 (0.007)	370 0.003)^{sh}
C20	10.211	1.047	200 (0.003)	238 (0.012)			333 (0.0025)	
C21	10.698	1.034	200 (0.014)	238 (0.012)			328 (0.015)	
C22	11.064	1.026	200 (0.013)	240 (0.005)^{sh}			323 (0.005)	
C23	11.361	1.017	200 (0.010)	233 (0.004)			318 (0.004)	
C24	11.561	1.048	200 (0.010)	238 (0.008)			323 (0.009)	
C25	12.119	1.028	200 (0.007)	238 (0.006)			328 (0.008)	
C26	12.467	1.034	200 (0.011)	238 (0.004)			323 (0.003)	
C27	12.903	1.021		238 (0.038)			309 (0.037)	
C28	13.181	1.011	200 (0.004)	238 (0.003)			318 (0.003)	
C29	13.338	1.039	200 (0.008)	233 (0.007)			313 (0.007)	
C30	13.870	1.214	200 (0.008)	238 (0.003)	270 (0.002)^{sh}		323 (0.003)	
C31	16.841		200 (0.008)	228 (0.005)			318 (0.003)	

* retention factor: $k' = (t_i - t_0) / t_0$; t_0 = 1.93 min.

** selectivity: $\alpha = k'_{i+1} / k'_i$, where i - peak number.

*** The absorbance values (in brackets) are obtained using PDA detector and are baseline corrected.

sh shoulder in the UV spectra rather then a maximum.

Table E-3. Retention (capacity) factor and selectivity for the HPLC separation together with UV data for the compounds formed during the enzymatic treatment of CM.

Peak	k'*	α**	λ (nm) and corresponding absorbance values***					
0	2.010	1.394	204 (0.011)	240^{v} (0.003)	251 (0.004)	284^{v} (0.001)	304 (0.002)	
1	2.218	1.175	200 (0.016)		246^{sh} (0.003)	290 (0.013)		360 (0.001)
2	2.606	1.348	200 (0.024)		260^{v} (0.001)	289 (0.002)		
3	3.514	1.707	200 (0.019)		252^{v} (0.002)	271 (0.002)	301^{v} (0.001)	333 (0.001)
4	6.000	1.120	200 (0.046)		248^{sh} (0.007)	285^{sh} (0.003)		324 (0.005)
5	6.720	1.077	200 (0.018)		248^{sh} (0.006)	286^{v} (0.003)		338 (0.005)
6	7.238	1.193	202 (0.009)	233^{v} (0007)	249 (0.008)	283^{v} (0.003)	317^{sh} (0.005)	345 (0.006)
7	8.635	1.019	200^{v} (0.055)	205(0.055)	231^{v} (0.018)	246 (0.020)	275^{v} (0.005)	331 (0.012)
8	8.799	1.037	200 (0.057)	226^{v} (0.007)	242 (0.008)	265^{v} (0.003)		325 (0.009)
9	9.128	1.079	200 (0.022)			276 (0.001)		324 (0.002)
10	9.853	1.053	200^{v} (0.036)	207 (0.041)	242^{sh} (0.009)	260^{v} (0.003)	273 (0.004)	352 (0.001)
11	10.378	1.034	200 (0.006)					330 (0.001)
12	10.730	1.027	203 (0.013)			281^{v} (0.002)		325 (0.003)
13	11.019	1.101	200^{v} (0.110)	205 (0.117)	231^{v} (0.039)	250 (0.050)	276^{v} (0.010)	340 (0.033)
14	12.132	1.006	200^{v} (0.011)	206 (0.012)	243^{sh} (0.008)	279^{sh} (0.001)		
15	12.210	1.038	200 (0.039)	223^{v} (0.020)	243 (0.025)	269^{v} (0.007)		337 (0.028)
16	12.673	1.058	200 (0.024)	230^{sh} (0.010)		268^{v} (0.004)		323 (0.008)
17	13.404	1.055	200 (0.010)	245^{sh} (0.003)		276^{sh} (0.002)		325 (0.003)
18	14.145		203 (0.013)	242^{sh} (0.005)		278^{v} (0.002)		339 (0.005)

* retention factor: $k' = (t_i - t_0) / t_0$; t_0 = 1.93 min.

** selectivity: $\alpha = k'_{i+1} / k'_i$, where i - peak number.

*** The absorbance values (in brackets) are obtained using PDA detector and are baseline corrected.

sh shoulder in the UV-spectra rather then a maximum.

v valley in the UV spectra

E.2 Mass Spectrometry

Table E-4. Mass spectrometry data for SIN, SA and the products of their transformation by the polyphenol oxidase from *T. versicolor*

Compound Name	#	Mw	Characteristic ion fragmentation
sinapic acid	SA	224	121(68) 135(3) 149(7) 165(8) 180(19) 209(13) 224(100)(M^+) 225(14)
sinapine	SIN	310	104(31) 149(25) 167(30) 186(65) 207(12) 215(10) 251(20) 310(56)(M^+)
	I	446	123(3) 151(15) 154(11) 167(21) 181(100) 182(31) 220(17) 358(13) 402(5) 446(46)(M^+)
dehydrodisinapic acid dilatcone	II	446	129(10) 139(22) 154 (55) 167(100) 168(13) 181 (55) 182(16) 192(41) 220(12) 233(47) 248(90) 249(21) 358(15) 402(90) 446(25)(M^+)
	VII	400	44 (87) 57(19) 69(12) 71(10)154(8) 167(18) 181(100) 192 (11) 248(86) 400(46)(M^+) 402(11)
	VIII	388	57(35) 69(23) 181(100) 196(15) 388(11)(M^+)
	IX	388	69(40) 117(100) 129(16) 149(9) 181(48) 197(23) 232(23) 239(15) 247(12) 285(13) 315(14) 371(6) 388(10)(M^+)
2,6-dimethoxy-p-benzoquinone	XI	168	53(25) 59(24) 69(100) 80(47) 97(10) 110(4) 112(9) 125(12) 138(22) 168(51)(M^+)
	XII	458	139(5) 154(8) 167(36) 181(100) 182(12) 192(15) 197(10) 248(9) 358(14) 400(54) 458(10)(M^+)
	XIV	400	71(33) 117(25) 129(11) 181(100) 400(58)(M^+) 401(17)

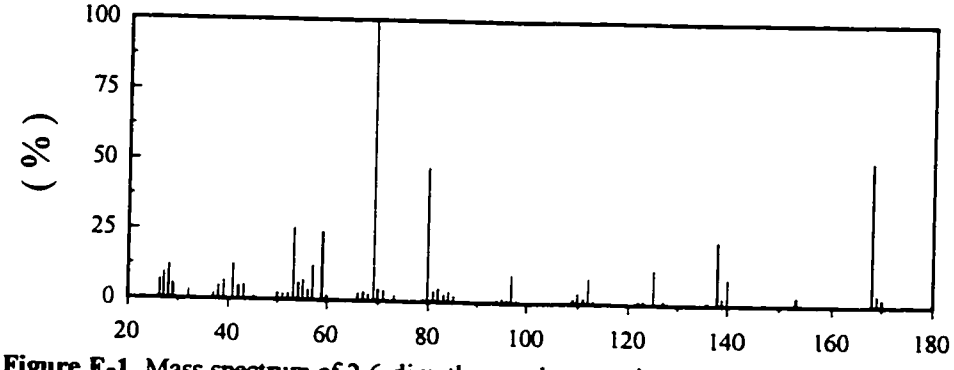


Figure E-1. Mass spectrum of 2,6-dimethoxy-p-benzoquinone obtained by the DIP method.

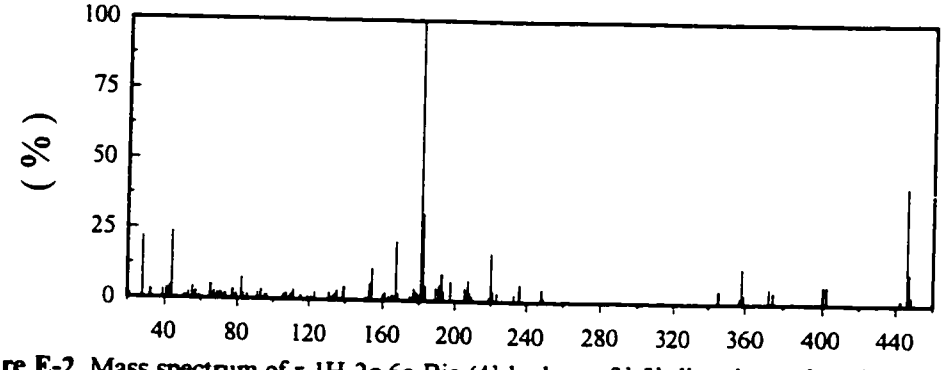


Figure E-2. Mass spectrum of r-1H-2c,6c-Bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione obtained by the DIP method.

Infra-red Spectrophotometry

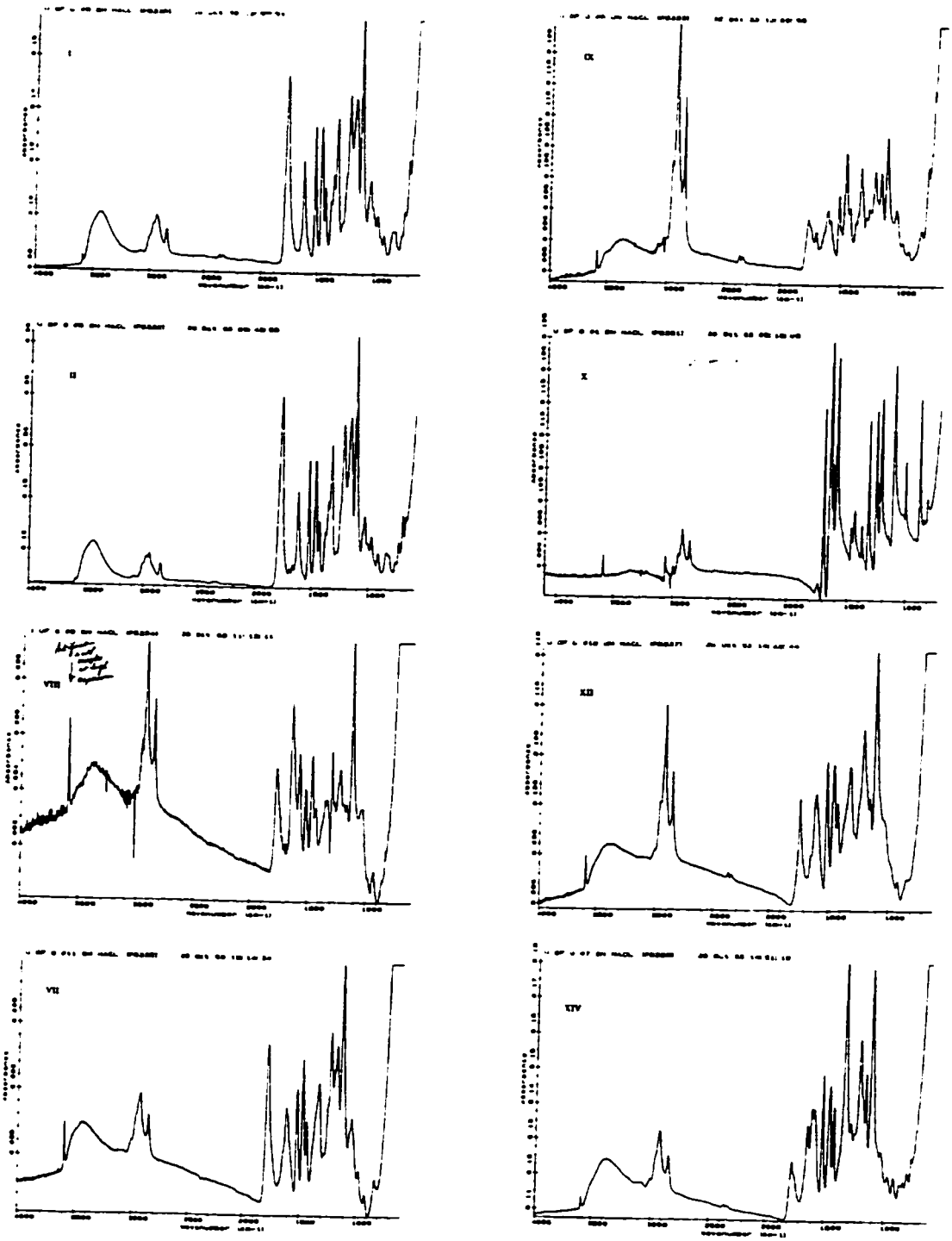


Figure E-3. IR spectra of the products from the enzymatic transformation of SA.

E.4 NMR spectrometry

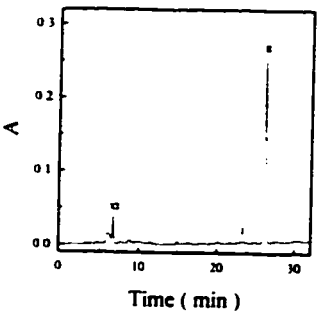


Figure E-4. HPLC chromatogram of the MeCl solution of the sample used for NMR analysis recorded at 280 nm.

Table E-5. ^{1}H and ^{13}C chemical shifts values for r-1H-2c,6c-Bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione.

C/H	δH	mult.	J (Hz)	δC
1, 5	3.87	d	1.3	48.91
2, 6	5.82	s	-	82.37
4, 8	-	-	-	175.21
Ar-H	6.53	s	-	102.16
Ar-OH	5.59	s	-	135.87
Ar-OCH_3	-	-	-	148.02
Ar-C	-	-	-	129.59
OCH_3	3.79	s	-	56.87

mult, multiplicity (coupling pattern); s, singlet; d, doublet.

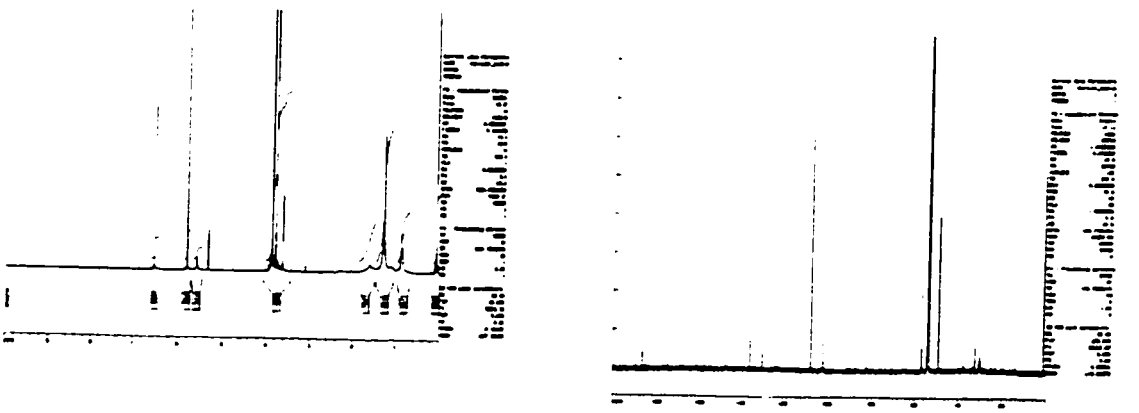


Figure E-5. ^{1}H (a) and ^{13}C (b) NMR spectra of the products formed during Phase I of SA transformastion.

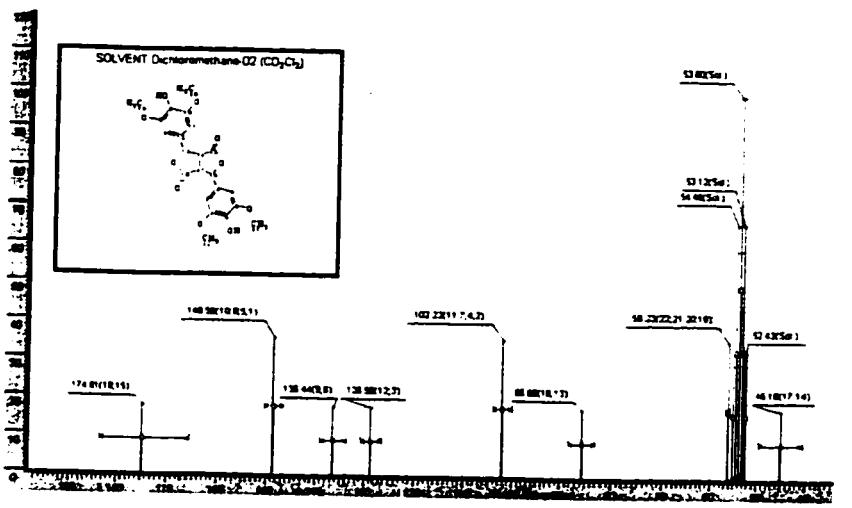


Figure E-6. NMR ^{13}C spectra of r-1H-2c,6c-Bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione generated by the computer package ACD CNMR.

E.5 X-Ray Crystallography

EXPERIMENTAL

1. Data Collection

A crystal of $O_{10}C_{22}H_{22}$ having approximate dimensions of 0.2, 0.2, 0.2 mm was mounted on a glass capillary. All the measurements were made on a Nonius diffractometer with Cu Kα radiation.

Cell constants and an orientation matrix for data collection, were obtained from least-squares refinement using the setting angles of 25 reflections in the range $100<2\Theta<140$ corresponding to a monoclinic cell with dimensions:

a = 7.8896(4)
b = 19.1566(7)
c = 13.6213(4)
β = 94.255(3)

For Z=4 and FW=446.41, the calculated density is 1.444g/cm3. Based on the systematic absences, the space group was determined to be $P2_1/a$.

The data was collected at room temperature using the omega2theta scan technique to a maximum 2Θ value of 140.0 degrees.

2. Data Reduction:

A total of 3907 reflections were collected. The unique set contains only 3900 reflections. The standards were measured after every 150 reflections. No crystal decay was noticed. The data were corrected for Lorentz and polarisation effects. No absorption correction was made.

3. Solution and refinement:

The structure was solved by direct methods. All the atoms were refined anisotropically except the hydrogen. The hydrogen atoms were found by differences Fourier map. The final cycle of full matrix least-squares refinement was based on 3198 observed reflections ($I>2.5\sigma(I)$) and 378 variable parameters. Weights based on counting statistics were used. The maximum and minimum peaks on the final differences Fourier map corresponded to 0.380 and - 0.330 e $Å^{-3}$, respectively.

All the calculations were performed using the NRCVAX crystallographic software package.

EXPERIMENTAL DETAILS

Empirical formula	$O_{10}C_{22}H_{22}$
Formula weight	446.41
Crystal shape	cube
Crystal dimensions (mm)	0.2, 0.2, 0.2
Crystal system	monoclinic
No. Reflections used for unit cell dimension (2Θ range)	35 100 - 140
Lattice parameters:	a = 7.8896(4) b = 19.1566(7) c = 13.6213(4) β = 94.255(3)
Space group	P 21/a
Z value	4
D_{calc} (g cm^{-3})	1.444
F(000)	939.38
μ (mm^{-1})	0.69
λ (Å)	1.54056
No. of reflections measured	3907
No. of reflections unique	3900
No. of reflections observed	3198
No. of atoms	54
No. of variables	378
R_f(sign refl)	0.059
R_w (sign refl)	0.072
R_f(all refl)	0.068
R_w (all refl)	0.073
Goodness of fit	3.24
Last difference Fourier map:	
max peak	0.380
min peak	-0.330

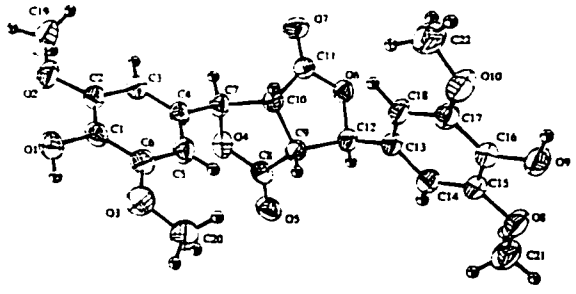


Figure E-7. ORTEP view of the molecule of: *r-1H-2c,6c-Bis-(4'-hydroxy-3',5'-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane-4,8-dione* showing labeling.

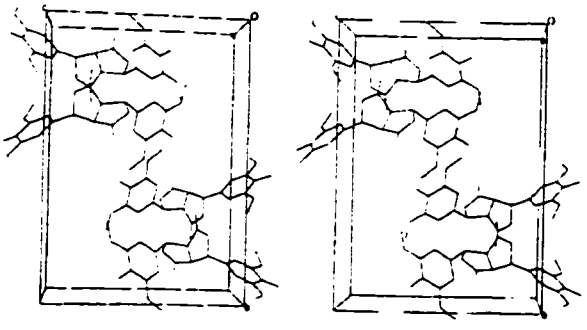


Figure E-8. Stereo packing diagram representing crystal of dehydrodisinapic acid dilactone

Table E-6. Atomic Parameters x,y,z and E.S.Ds. refer to the last digit printed.

	x	y	z	Biso
O1	0.36515 (21)	0.42043 (9)	0.36490 (12)	4.03 (7)
O2	0.24387 (24)	0.29411 (10)	0.33043 (13)	4.55 (8)
O3	0.28433 (24)	0.49576 (9)	0.51768 (14)	4.73 (8)
O4	-0.24958 (19)	0.34351 (9)	0.60677 (11)	3.55 (7)
O5	-0.39106 (21)	0.42816 (9)	0.67854 (13)	4.16 (7)
O6	-0.21828 (19)	0.28004 (7)	0.85928 (10)	3.11 (6)
O7	-0.09838 (22)	0.19346 (8)	0.78048 (12)	3.93 (7)
O8	-0.19197 (24)	0.49953 (9)	1.16312 (12)	4.60 (8)
O9	0.11258 (23)	0.45468 (9)	1.21639 (12)	4.39 (7)
O10	0.28314 (23)	0.36850 (10)	1.10622 (13)	4.93 (8)
C1	0.2586 (3)	0.39249 (12)	0.42989 (16)	3.38 (8)
C2	0.1935 (3)	0.32641 (12)	0.41273 (16)	3.32 (8)
C3	0.0820 (3)	0.29804 (11)	0.47700 (16)	3.22 (9)
C4	0.0392 (3)	0.33616 (11)	0.55820 (15)	3.12 (8)
C5	0.1066 (3)	0.40223 (12)	0.57625 (17)	3.60 (9)
C6	0.2150 (3)	0.43077 (12)	0.51138 (17)	3.47 (9)
C7	-0.0882 (3)	0.30615 (11)	0.62366 (15)	3.03 (8)
C8	-0.2752 (3)	0.38792 (11)	0.68215 (16)	3.13 (8)
C9	-0.1404 (3)	0.37738 (10)	0.76548 (15)	2.77 (7)
C10	-0.0431 (3)	0.31411 (10)	0.73447 (15)	2.65 (7)
C11	-0.1170 (3)	0.25486 (11)	0.79116 (15)	2.80 (8)
C12	-0.2179 (3)	0.35611 (11)	0.86234 (15)	2.90 (8)
C13	-0.1243 (3)	0.38079 (11)	0.95593 (15)	3.01 (8)
C14	-0.2056 (3)	0.42790 (12)	1.01430 (16)	3.28 (8)
C15	-0.1246 (3)	0.45255 (12)	1.10079 (16)	3.36 (9)
C16	0.0385 (3)	0.43030 (12)	1.13082 (15)	3.28 (9)
C17	0.1205 (3)	0.38364 (12)	1.07117 (16)	3.42 (9)
C18	0.0403 (3)	0.35929 (12)	0.98404 (16)	3.42 (9)
C19	0.1841 (4)	0.22560 (18)	0.31020 (24)	6.12 (15)
C20	0.2382 (4)	0.53981 (13)	0.59414 (21	4.50 (11)
C21	-0.3611 (4)	0.52297 (16)	1.13745 (21)	5.34 (13)
C22	0.3671 (4)	0.31245 (18)	1.06394 (22)	5.64 (14)
H3	0.045 (3)	0.2504 (12)	0.4659 (17)	4.0 (5)
H5	0.099 (3)	0.4312 (14)	0.6333 (19)	5.5 (7)
H7	-0.118 (3)	0.2554 (12)	0.6085 (17)	3.6 (5)
H9	-0.079 (3)	0.4184 (12)	0.7721 (16)	3.3 (5)
H10	0.0771 (25)	0.3148 (10)	0.7507 (14)	2.4 (4)
H12	-0.334 (3)	0.3731 (11)	0.8573 (15)	2.8 (4)
H14	-0.313 (3)	0.4405 (12)	0.9955 (18)	4.1 (5)
H18	0.101 (3)	0.3243 (12)	0.9428 (17)	4.0 (5)
H19A	0.061 (5)	0.2230 (17)	0.3007 (24)	8.5 (9)
H19B	0.214 (5)	0.2106 (20)	0.249 (3)	10.8(12)
H19C	0.231 (5)	0.1956 (20)	0.371 (3)	11.4 (12)
H20A	0.276 (4)	0.5225 (15)	0.6628 (21)	6.2 (7)
H20B	0.296 (3)	0.5839 (14)	0.5835 (20)	5.6 (7)
H20C	0.108 (3)	0.5448 (13)	0.5976 (20)	5.2 (6)
H21A	-0.374 (3)	0.5435 (14)	1.0729 (20)	5.6 (7)
H21B	-0.383 (4)	0.5562 (15)	1.1832 (21)	6.5 (8)
H21C	-0.434 (3)	0.4815 (14)	1.1357 (19)	5.2 (6)
H22A	0.461 (4)	0.3099 (15)	1.1046 (20)	6.2 (7)
H22B	0.293 (4)	0.2708 (14)	1.0675 (21)	6.3 (7)
H22C	0.362 (4)	0.3166 (17)	0.9963 (24)	7.8 (9)
OH1	0.375 (5)	0.4665 (17)	0.376 (3)	8.9 (10)
OH9	0.199 (3)	0.4338 (14)	1.2371 (21)	5.7 (7)

Table E-7. Atomic Bond Distances (Å)

O1-C1	1.374 (3)	C9-C12	1.549 (3)
O1-OH1	0.90 (3)	C9-H9	0.924 (22)
O2-C2	1.365 (3)	C10-C11	1.514 (3)
O2-C19	1.415 (4)	C1O-HIO	0.958 (20)
O3-C6	1.360 (3)	C12-C13	1.502 (3)
O3-C2O	1.409 (3)	C12-HI2	0.967 (21)
O4-C7	1.464 (3)	C13-C14	1.391 (3)
O4-C8	1.360 (3)	C13-C18	1.388 (3)
O5-C8	1.194 (3)	C14-C15	1.381 (3)
O6-C11	1.3571 (25)	C14-HI4	0.896 (24)
O6-C12	1.4578 (25)	C15-C16	1.388 (3)
O7-C11	1.196 (3)	C16-C17	1.399 (3)
O8-C15	1.371 (3)	C17-C18	1.384 (3)
O8-C21	1.427 (3)	C18-HI8	1.020 (23)
O9-C16	1.348 (3)	C19-HI9A	0.97 (4)
O9-OH9	0.82 (3)	C19-HI9B	0.93 (4)
O1O-C17	1.366 (3)	C-19-HI9C	1.05 (4)
O1O-C22	1.407 (3)	Ç2O-H2OA	1.02 (3)
C1-C2	1.380 (3)	C2O-H2OB	0.98 (3)
C1-C6	1.395 (3)	C2O-H2OC	1.03 (3)
C2-C3	1.396 (3)	C21-H21A	0.96 (3)
C3-C4	1.388 (3)	C21-H21B	0.92 (3)
C3-H3	0.966 (23)	C21-H21C	0.98 (3)
C4-C5	1.388 (3)	C22-H22A	0.89 (3)
C4-C7	1.506 (3)	C22-H22B	0.99 (3)
C5-C6	1.387 (3)	C22-H22C	0.92 (3)
C5-H5	0.96 (3)	H19A-HI9B	1.46 (6)
C7-C1O	1.532 (3)	H21A-H21B	1.53 (4)
C7-H7	1.018 (23)	H21A-H21C	1.56 (4)
C8-C9	1.511 (3)	H22B-H22C	1.44 (4)
C9-C1O	1.512 (3)		

Table E-8. Atomic Bond Angles (°)

C1-O1-OH1	109.1 (22)	C9-C12-HI2	106.4 (12)
C2-O2-C19	117.86 (20)	C13-C12-HI2	110.9 (12)
C6-O3-C2O	118.04 (18)	C12-C13-C14	118.11 (19)
C7-O4-C8	111.31 (16)	C12-C13-C18	122.11 (18)
C11-O6-C12	112.01 (15)	C14-C13-C18	119.76 (20)
C15-O8-C21	116.96 (19)	C13-C14-C15	120.38 (2O)
C16-O9-OH9	115.O (19)	C13-C14-HI4	118.4 (16)
C17-O1O-C22	118.52 (19)	C15-C14-HI4	121.2 (16)
O1-C1-C2	119.11 (2O)	O8-C15-C14	125.2O (21)
O1-C1-C6	12O.45 (21)	O8-C15-C16	114.37 (2O)
C2-C1-C6	12O.44 (19)	C14-C15-C16	12O.44 (19)
O2-C2-C1	115.3O (19)	O9-C16-C15	118.78 (19)
O2-C2-C3	125.O2 (21)	O9-C16-C17	122.29 (21)
C1-C2-C3	119.67 (2O)	C15-C16-C17	118.94 (2O)
C2-C3-C4	119.78 (2O)	O1O-C17-C16	113.31 (19)
C2-C3-H3	117.8 (14)	O1O-C17-C18	125.88 (2O)
C4-C3-H3	122.2 (14)	C16-C17-C1S	12O.77 (21)
C3-C4-C5	12O.56 (19)	C13-C18-C17	119.7O (19)
C3-C4-C7	118.91 (19)	C13-C18-HI8	121.O (13)
C5-C4-C7	12O.46 (19)	C17-C18-HI8	119.3 (13)
C4-C5-C6	119.54 (21)	O2-C19-HI9A	112.9 (19)
C4-C5-H5	128.6 (16)	O2-C19-HI9B	111.O (24)
C6-C5-H5	111.5 (16)	O2-C19-HI9C	1O5.2 (22)
O3-C6-C1	114.2O (19)	H19A-C19-HI9B	1OO (3)
O3-C6-C5	125.79 (21)	H19A-C19-HI9C	111 (3)
C1-C6-C5	12O.OO (21)	H19B-C19-HI9C	115 (3)
O4-C7-C4	1O9.35 (17)	O3-C2O-H2OA	114.2 (16)
O4-C7-C1O	1O4.OO (16)	O3-C2O-H2OB	1O5.O (16)
O4-C7-H7	1O4.4 (13)	O3-C2O-H2OC	113.6 (15)
C4-C7-C1O	115.38 (18)	H2OA-C2O-H2OB	1O8.1 (23)
C4-C7-H7	113.6 (13)	H2OA-C2O-H2OC	1O2.4 (22)
C1O-C7-H7	1O9.2 (13)	H2OB-C2O-H2OC	113.8 (22)
O4-C8-O5	121.94 (21)	O8-C21-H21A	112.8 (16)
O4-C8-C9	11O.35 (18)	O8-C21-H21B	1O5.9 (18)
O5-C8-C9	127.71 (21)	O8-C21-H21C	1O6.8 (15)
C8-C9-C1O	1O3.95 (17)	H21A-C21-H21B	1O9.O (24)
C8-C9-C12	111.98 (17)	H21A-C21-H21C	1O6.6 (22)
C8-C9-H9	1O7.1 (13)	H21B-C21-H21C	115.9 (23)
C1O-C9-C12	1O5.53 (16)	O1O-C22-H22A	1OO.4 (18)
C1O-C9-H9	115.8 (13)	O1O-C22-H22B	1O7.3 (17)
C12-C9-H9	112.3 (13)	O1O-C22-H22C	11O.8 (2O)
C7-C1O-C9	1O5.63 (17)	H22A-C22-H22B	112.8 (25)
C7-C1O-C11	11O.96 (17)	H22A-C22-H22C	126 (3)
C7-C1O-H1O	112.5 (12)	H22B-C22-H22C	97 (3)
C9-C1O-C11	1O3.64 (16)	C19-HI9A-HI9B	38.6 (21)
C9-C1O-H1O	116.O (12)	C19-HI9B-HI9A	4O.9 (22)
C11-C1O-H1O	1O7.7 (12)	C21-H21A-H21B	34.5 (14)
O6-C11-O7	121.12 (19)	C21-H21A-H21C	37.1 (14)
O6-C11-C1O	11O.54 (17)	H21B-H21A-H21C	62.8 (18)
O7-C11-C1O	128.32 (19)	C21-H21B-H21A	36.5 (15)
O6-C12-C9	1O3.81 (15)	C21-H21C-H21A	36.3 (14)
O6-C12-C13	1O9.81 (17)	C22-H22B-H22C	39.3 (17)
O6-C12-HI2	1O9.5 (12)	C22-H22C-H22B	42.9 (18)
C9-C12-C13	116.O9 (17)		

Appendix F

Listing of the Scientist® model file for the modeling of canola meal system

```
//*******************************
// MicroMath Scientist Model File
// written by Karol Lacki
// created on February 1997
// last modified on: March 26, 1997
//*******************************
// Model describing the enzymatic process of a decrease in phenolic content in canola meal or similar
// systems
// based on the extraction of the solute from two spherical particles with radii R1 and R2, and diffusivities
// Deff and D2eff
// Assuming Langmuir type of equilibrium between surface and bulk concentrations
// Taking into consideration the solvent uptake and matrix porosity.
//*******************************
// T - time [h]
// Ms - mass of solid [g]
// Vtot - volume of bulk liquid [mL]
// Venz - volume of enzyme added [mL]
// P - pressure [Pa]
// ro - density of bulk liquid [g/mL^3]
// ros - density of solid [g/cm^3]
// Vsys - total volume of the reactor [mL]
// enzyme0 - concentration of the enzyme stock solution [nkat/mL]
// Tem - temperature of the process [K]
// Temstar - reference temperature [K]
// b0 - beta, proportionality factor to express enzyme concentration in moles [mol/mL]
// m1 - mass fraction of particles with diameter R [-]
// R, R2 - radi of particles [cm]
// eps - porosity of the solid [-]
// uptake - liquid uptake by the solid [mL/g]
// wl0 - initial mas concentrtion of the solute in the bulk liquid [g/mL]
// C0ini - inital concntration of the solute in the solid [g/g]
// Xini - initial volume fraction of oxygen in the gas phase [-]
// z - concentration of the added enzyme in % of the stock solution
//Independent variables
IndVars: T,Vtot
//Dependent variables
DepVars:cs,cs1,cg1,wl1,Yi,cp1, enzyme
//Parameters
Params: E3,Eo2,Eio2,Esae,Epr
// Physico-chemical properties of the system
ro=1.0
```

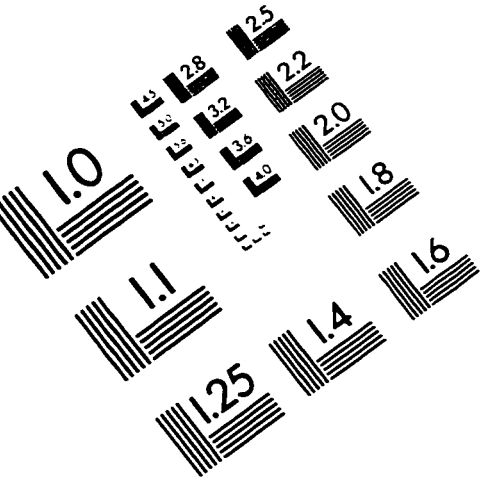
```
ros=1.0
R=0.0251
R2=R
m1=0.946
m2=1-m1
uptake=2.7
eps=0
enzyme0=40
b0=1.25E-4
Temstar=323
Rg=8.314
Mww=18
Mwo2=32
Mwsae=407
//Process conditions
Vsys=14
Ms=0.5
Vtot=5.0
Venz=0.5
Xini=0.21
Tem=323
P=101.325e3
z=10
wl0=0
C0initial=20.136*1e-3
C0ini=1
C0=C0initial*C0ini
C02=C0
//Condition for uptake Vu: Uptake can not be bigger than the continuous phase
Vu1=uptake*Ms
Vu2=Vtot-Vu1
Vu=IFLTZERO(Vu2,Vtot,Vu1)
//Values of the parameters at Temstar
Defft1=0.0006259
D2efft1=0.0000117817
KL1=0.0034794
kl2=0.038792
k30=0.13655
KO20=1.67e-21
KIO20=2.56e-6
KSAE0=7.7084e-6
Kpr0=1.1782e-5
P2=0.013183
P3=0.00036665
stoich=0.0004
KLA=100
//Extra equations
Vl=Vtot
DIL=z/100
//Definition of overall partition coefficient, Kiso=beta1; Biso=beta
A=(eps*ro/ros+Vu*ro/Ms)*Mwsae
beta=1/(A+1/((KL1+wl*Mwsae)^2/(KL1*kl2*Mwsae)))
beta1=1/(A+kl2*Mwsae/(KL1+wl*Mwsae))
```

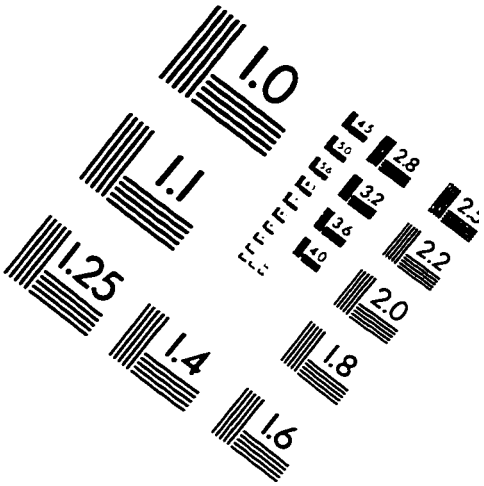
```
//Calculations of dynamic viscosities of water
miu1=((-0.0065007164-1.4070345e-5*Temstar)/(1-0.0045557637*Temstar))^2
miu2=((-0.0065007164-1.4070345e-5*Tem)/(1-0.0045557637*Tem))^2
//Calculation of diffusivities based on Wilke and Chang
Deff=Defft1*Tem/Temstar*(miu1/miu2)
D2eff=D2efft1*Tem/Temstar*(miu1/miu2)
//Calculations of rate constants
k3=k30*exp(-E3/Rg*(1/Tem-1/Temstar))
Ko2=Ko20*exp(-Eo2/Rg*(1/Tem-1/Temstar))
Kio2=Kio20*exp(-Eio2/Rg*(1/Tem-1/Temstar))
Ksae=Ksae0*exp(-Esae/Rg*(1/Tem-1/Temstar))
Kpr=Kpr0*exp(-Epr/Rg*(1/Tem-1/Temstar))
//Calculations of Henry constant for given process temperature: GIVEN IN Pa
dumy=3.71814+5.59617e3/Tem-1.049668e6/Tem^2
h=exp(dumy)*101.325e3
//Calculations of initial oxygen concentration and constant nitrogen concentration in moles per cm3
//of liquid
cg0=ro*Xini*P/(h*Mww-Xini*P*(Mww-Mwo2))
temporary=Tem-253
cgini=IFLTZERO(temporary,0,cg0)
//Enzyme deactivation
ke1=1.2015e14*Tem*exp(-118392/Rg/Tem)
ke2=3.8573e13*Tem*exp(-110652/Rg/Tem)
ke3=1.3071e18*Tem*exp(-137013/Rg/Tem)
enzyme=enzyme0*dil*(exp(-(ke1+ke2)*t)-ke1/(ke3-ke1-ke2)*(exp(-ke3*t)-exp(-(ke1+ke2)*t)))*Venz/Vl
//Definition of a degree of enzyme/meal saturation
ps=1/(1+(enzyme/(p2*1000))^(-p3*1000))
//**********************************
//Help equations
Ml=ro*Vl
Vg=Vsys-Ms*ros-Vtot
zeta=IFEQZERO (T,0,1)
n0=11
nf=500
kn1=Deff*(n*PI/R)^2
kn2=D2eff*(n*PI/R2)^2
f1=exp(-t*kn1)
f1j=exp(-t*kn2)
//Differential equations
//First particle
fi1'=-Deff*(PI/R)^2*(1)^2*fi1+1/beta*wl'
fi2'=-Deff*(PI/R)^2*(2)^2*fi2+1/beta*wl'
fi3'=-Deff*(PI/R)^2*(3)^2*fi3+1/beta*wl'
fi4'=-Deff*(PI/R)^2*(4)^2*fi4+1/beta*wl'
fi5'=-Deff*(PI/R)^2*(5)^2*fi5+1/beta*wl'
fi6'=-Deff*(PI/R)^2*(6)^2*fi6+1/beta*wl'
fi7'=-Deff*(PI/R)^2*(7)^2*fi7+1/beta*wl'
fi8'=-Deff*(PI/R)^2*(8)^2*fi8+1/beta*wl'
fi9'=-Deff*(PI/R)^2*(9)^2*fi9+1/beta*wl'
fi10'=-Deff*(PI/R)^2*(10)^2*fi10+1/beta*wl'
fi11'=-Deff*(PI/R)^2*(11)^2*fi11+1/beta*wl'
//Second particle
fij1'=-D2eff*(PI/R2)^2*(1)^2*fij1+1/beta*wl'
```

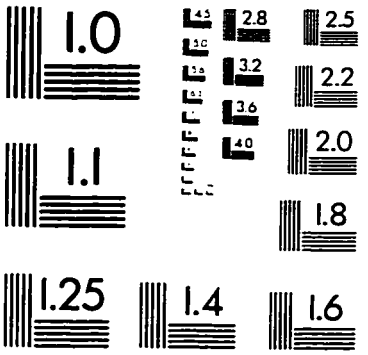
```
      fij2'=-D2eff*(PI/R2)^2*(2)^2*fij2+1/beta*wl'
      fij3'=-D2eff*(PI/R2)^2*(3)^2*fij3+1/beta*wl'
      fij4'=-D2eff*(PI/R2)^2*(4)^2*fij4+1/beta*wl'
      fij5'=-D2eff*(PI/R2)^2*(5)^2*fij5+1/beta*wl'
      fij6'=-D2eff*(PI/R2)^2*(6)^2*fij6+1/beta*wl'
      fij7'=-D2eff*(PI/R2)^2*(7)^2*fij7+1/beta*wl'
      fij8'=-D2eff*(PI/R2)^2*(8)^2*fij8+1/beta*wl'
      fij9'=-D2eff*(PI/R2)^2*(9)^2*fij9+1/beta*wl'
      fij10'=-D2eff*(PI/R2)^2*(10)^2*fij10+1/beta*wl'
      fij11'=-D2eff*(PI/R2)^2*(11)^2*fij11+1/beta*wl'
      // Summ evaluation
      //First particle
      f1sum=SUMMATION(1,nf,exp(-t*Deff*((n*PI/R)^2)),n)
      f2sum=SUMMATION(1,nf,(exp(-t*Deff*((n*PI/R)^2))/(n*n)),n)
      fisum=fi1+fi2+fi3+fi4+fi5+fi6+fi7+fi8+fi9+fi10+fi11
      fisum2=fi1+1/(2)^2*fi2+1/(3)^2*fi3+1/4^2*fi4+1/25*fi5+1/36*fi6+1/49*fi7+1/64*fi8+1/81*fi9+
c     1/100*fi10+1/11^2*fi11
      //Second particle
      f1jsum=SUMMATION(1,nf,exp(-t*D2eff*((n*PI/R2)^2)),n)
      f2jsum=SUMMATION(1,nf,(exp(-t*D2eff*((n*PI/R2)^2))/(n*n)),n)
      fijsum=fij1+fij2+fij3+fij4+fij5+fij6+fij7+fij8+fij9+fij10+fij11
      fijsum2=fij1+1/(2)^2*fij2+1/(3)^2*fij3+1/4^2*fij4+1/25*fij5+1/36*fij6+1/49*fij7+1/64*fij8+1/81*fij9+
c     1/100*fij10+1/11^2*fij11
      //General summations
      fnsum=SUMMATION(1,n0,1/n^2,n)/(PI^2/6)
      fnsumf=SUMMATION(1,nf,1/n^2,n)/(PI^2/6)
      fnsum4=SUMMATION(1,n0,1/n^4,n)/(PI^4/90)
      //calculations of the equilibrium concentration of oxygen
      cgstar=ro*Yi*P/(h*Mww-Yi*P*(Mww-Mwo2))
      //model equations
      vmax=1000*b0*ps*k3*enzyme
      Yi'=-Rg*Tem/P*(Vtot/(Vg*1e-6))*kla*(cgstar-cg)
      cg'=kla*(cgstar-cg)-
c     1/(stoich*10000)*(vmax*cg*(Ksae*cp+Kpr*wl)/((cg+Ko2)*(Ksae*cp+Kpr*wl)+Ksae*Kio2*Kpr+
c     Ksae*Kpr*cg))
      cp'=-(vmax*cg*(Ksae*cp-Kpr*wl)/((cg+Ko2)*(Ksae*cp+Kpr*wl)+Ksae*Kio2*Kpr+Ksae*Kpr*cg))
      wl'=(1/Mwsae*6*Ms/Ml*(m1*(1/R^2)*Deff*((C0-wl0/beta1)*f1sum-fisum)+m2*(1/R2^2)*D2eff*((C02-
c     wl0/beta1)*f1jsum-fijsum))-vmax*cg*Kpr*wl/((cg+Ko2)*
c     (Ksae*cp+Kpr*wl)+Ksae*Kio2*Kpr+Ksae*Kpr*cg))/(1+1/Mwsae*1/beta*Ms/Ml*(1-fnsum))
      cs11=m1*(wl/beta1+6/(PI^2)*((C0-wl0/beta1)*(f2sum+PI^2/6*(1-fnsumf))-fisum2-
c     zeta*wl'/beta*(R*PI)^2/90/Deff*(1-fnsum4)))
      cs22=m2*(wl/beta1+6/(PI^2)*((C02-wl0/beta1)*(f2jsum+PI^2/6*(1-fnsumf))-fijsum2-
c     zeta*wl'/beta*(R2*PI)^2/90/D2eff*(1-fnsum4)))
      cs=(cs11+cs22)/C0initial
      cs1=m1*6/(PI^2)*C0*(f2sum+PI^2/6*(1-fnsumf))/C0initial+m2*6/(PI^2)*C02*(f2jsum+PI^2/6*(1-
c     fnsumf))/C0initial
      cg1=cg/cg0
      wl1=wl*Mwsae/C0initial
      cp1=cp/C0initial*Mwsae
      //initial conditions
      t=0
      fi1=0
      fi2=0
      fi3=0
```

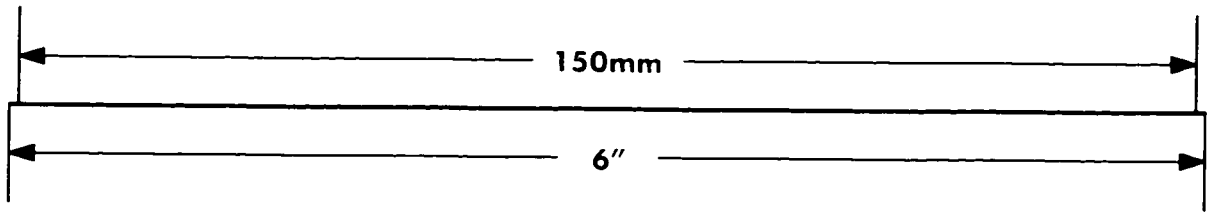
```
fi4=0
fi5=0
fi6=0
fi7=0
fi8=0
fi9=0
fi10=0
fi11=0
fij1=0
fij2=0
fij3=0
fij4=0
fij5=0
fij6=0
fij7=0
fij8=0
fij9=0
fij10=0
fij11=0
wl=wl0
cg=cgini
cp=0
Yi=Xini
//Parameters
E3=41753
Eo2=-200000
Eio2=92252
Esae=-51249
Epr=200000
******
```

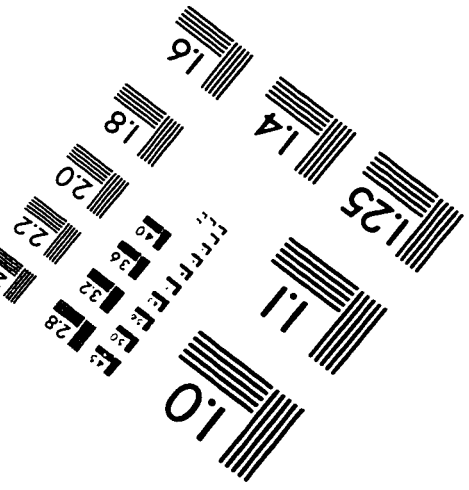
IMAGE EVALUATION TEST TARGET (QA-3)

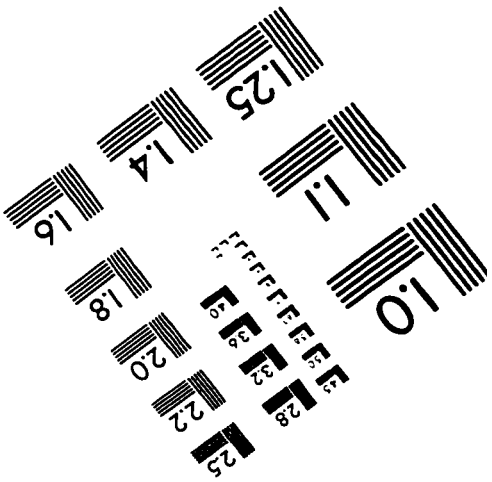












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